BK_{Ca} CHANNEL EXPRESSION MEDIATES SEASONAL AUDITORY HAIR CELL PLASTICITY

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Vertebrates displaying seasonal shifts in reproductive behavior offer the opportunity to investigate bidirectional plasticity in sensory function. The plainfin midshipman (*Porichthys notatus*) teleost fish exhibits seasonal, steroid-dependent plasticity in frequency encoding by eighth nerve auditory afferents. To what extent changes in afferent encoding are due to plasticity of the target auditory hair cells, inclusive of molecular mechanisms of auditory encoding, were previously unanswered. Evoked potentials recorded *in vivo* from auditory hair cells of the sacculus, the primary division of the inner ear used for hearing in this and many teleosts, revealed frequency-dependent plasticity in auditory hair cell function in both males and females. Auditory thresholds were lower in reproductive compared to non-reproductive animals with the greatest differences occurring at higher frequencies corresponding to high-energy harmonics of vocalizations. This plasticity correlated with seasonal fluctuations in circulating steroid levels suggesting that, like saccular afferents, plasticity at the hair cell level may be steroid-dependent. Large-conductance calcium-activated potassium (BK_{Ca}) channels in non-mammalian vertebrates determine the electrical resonant properties of auditory hair cells with higher expression levels coinciding with increased best frequency. In teleosts, the pore-

forming α-subunit of BK_{Ca} channels is encoded by duplicate *slo1* genes resulting from whole genome duplication at the origin of teleosts. In midshipman, duplicate *slo1* genes were retained through tissue-specific subfunctionalization. Expression of at least one *slo1* gene is significantly upregulated in the auditory epithelium of reproductive animals compared to non-reproductives, supporting the hypothesis that increased *slo1* and BK_{Ca} channel expression facilitates higher resonant frequencies and decreased thresholds in hair cells during the reproductive season. *In vivo* manipulation of BK_{Ca} currents using either the broad-spectrum potassium channel antagonist tetraethylammonium chloride (TEA) or the BK_{Ca} channel-specific antagonist iberiotoxin (IbTx) induced frequency-dependent changes in auditory hair cell response thresholds on the order of naturally occurring seasonal plasticity. Reproductive animals treated with either antagonist had thresholds like those recorded in non-reproductives, consistent with the role of increased BK_{Ca} channel expression facilitating higher electrical resonance in non-mammalian vertebrate hair cells. We propose that changes in BK_{Ca} channel abundance are a primary mechanism for frequency tuning plasticity in auditory hair cells among vertebrates.

BIOGRAPHICAL SKETCH

Kevin was born in Bethlehem, Pennsylvania on April 20, 1982 to parents Carl and Kathy. As a wee lad his parents and paternal grandfather, Charles, nurtured Kevin's inner nerd with numerous trips to natural history museums to see "dead animals in glass cases" and aquariums even though the nearest one was in Camden, New Jersey and grandpa was left cursing "For the love of mud Marge" as he couldn't navigate his way back to Pennsylvania from its lesser neighboring state. Always a stickler for literal description of the natural world around him, Kevin enjoyed the great outdoors where again, with the aid of his grandfather, he spent many enjoyable afternoons "planting sticks" as he once described the free Arbor Day saplings planted on his grandfather's country estate. Kevin's love for the "ologies," especially the "bio-" flavor, was nurtured further at Liberty High school in Bethlehem, Pennsylvania where he was able to take multiple biology elective courses including genetics, human anatomy and physiology, and microbiology.

After two-and-a-half years majoring in Biology and Psychology at the gulag work camp known as Carnegie Mellon University in Pittsburgh, Pennsylvania where Kevin academically excelled in spite of difficult medical circumstances, he transferred to Lehigh University in Bethlehem, Pennsylvania where he majored in Behavioral Neuroscience. Having already spent a summer as an undergraduate working in the laboratory of Colin J. Saldanha, Ph.D, before transferring to Lehigh, Kevin continued working in Colin's lab for the next two-and-a-half years. It was in Colin's laboratory where Kevin learned such fundamental scientific principles as "work hard, play hard." One summer Colin drove a minivan full of undergraduates, including Kevin, to the annual meeting of the Society for Behavioral Neuroendocrinology in scenic Cincinnati, Ohio where, during a keynote lecture by one Andrew H. Bass, Ph.D., Kevin first heard about "singing fish."

Even though the talk was first thing in the morning and, let's face it, he probably fell asleep half way through it as he does every talk he attends, something must have piqued Kevin's interest that day because several years later he left the bright light of the electric star shining atop South Mountain in Bethlehem for the remote ivy-infested field station known as Cornell University to begin his graduate work in Dr. Bass's lab. After a little over five-and-a-half years, not that anyone counts these things or keeps institutional records for tracking purposes, Kevin composed the tome that lies before you, most likely in electronic form hardly befitting the use of the word "tome."

To Mom and Dad for their support and to my Grandfather Charles Rohmann who took me to see ‘dead animals in glass cases’ as a kid.

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My final thanks go to my "extended" family, both biological and other. My undergraduate advisor Colin J. Saldanha, known as "Big Mamma" to his students, has served as a mentor, a friend, and a maternal influence since I began work in his lab in 2002. To this day he still takes pride in my accomplishments and provides support when I struggle, often using his mind reading abilities to sense when I need help before I do. My biological parents Carl and Kathy Rohmann have been ever supportive of me as I've followed a path whose goals and objectives make little to no sense to them but that they know make me incredibly excited. My brother Andy has always been there to remind me how big of a dork I truly am.

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CHAPTER 1

INTRODUCTION: MIDSHIPMAN FISH AS A MODEL SYSTEM FOR THE STUDY OF AUDITORY PLASTICITY

Potassium channels in vertebrate auditory hair cells are responsible for conducting currents that convert the physical stimulus of sound waves to electrical signals that are then processed by the brain. Studies in mice and turtles show that calcium-activated, large conductance potassium (BK) channels are responsible for currents that contribute to the encoding of low-frequency sound by hair cells (Jones et al., 1999a; Adamson et al., 2002). BK channels contribute to hair cell resonance, the low frequency (<1kHz) membrane oscillations elicited by intracellular current injection (Fettiplace and Fuchs, 1999). Until now, little work has been done to investigate BK channel expression in vertebrates that use low frequency sound in a well-characterized behavior. The experiments reported in this dissertation test the hypothesis that BK channels contribute to frequency tuning in auditory hair cells in the peripheral auditory system of a vocal organism, and that changes in expression of these channels is responsible for seasonal plasticity of peripheral auditory encoding.

The male midshipman fish (*Porichthys notatus*) produces low frequency, multiharmonic vocalizations (fundamental frequency ~100 Hz) known as "hums" to attract reproductive females to nests. The primary organ of hearing in midshipman is the saccular division of the inner ear that shares functional properties with the turtle cochlea. Midshipman saccular nerve afferents, branches of the eighth nerve, show optimized responses to pure tones of 60-400 Hz (McKibben and Bass, 1999; Sisneros

and Bass, 2003; Sisneros et al., 2004a), falling within the range of frequencies electrically tuned for by the BK channel in turtles (Fettiplace and Fuchs, 1999). The saccular nerve of midshipman females show seasonal, steroid-dependent increases in the degree of temporal encoding of frequency (Sisneros and Bass, 2003; Sisneros et al., 2004a), suggesting possible shifts in the BK-dependent properties of saccular auditory hair cells. Such adaptive hearing changes would increase the ability of females to encode male song during the mating season. Given that vocal behavior is essential for successful reproduction in midshipman fish (Bass, 1996), midshipman provide a unique model in which the molecular basis of auditory plasticity can be established in the context of an acoustic stimulus that plays a crucial role in an animal's life history. The ease with which the saccule can be accessed makes the midshipman an excellent model in which to study *in vivo* auditory physiology in a completely intact inner ear. Additionally, I have identified two *slo1* genes in teleost fishes (Chapter 3) including midshipman and zebrafish, a model used to study auditory mechanotransduction (Nicolson, 2005). This provides a unique opportunity to combine molecular neurobiology and physiology to study the expression and physiological role of BK channels in an organism in which the pore-forming α -subunit is encoded by not one but two *slo1* genes in what is otherwise a small, yet complex and important, gene family within the broader group of potassium channels.

Vocal-acoustic System of Midshipman Fish

During the summer reproductive season, male midshipman nest in the intertidal zone along the pacific coast of North America, producing low frequency vocalizations

(fundamental frequency ~100Hz) known as hums to attract females to the nest (Bass, 1996). Reproductively active female midshipman display a robust, stereotyped positive phonotactic response to underwater playback of recorded or synthesized male hums (McKibben and Bass, 1998, 2001). The peripheral auditory system of midshipman is well suited for encoding sound within the frequency range of vocalizations. Best excitatory frequencies recorded from primary auditory afferents in the eighth nerve branch that innervates the saccular epithelium range from 60-250Hz, with units responding to frequencies as high as 400Hz (McKibben and Bass, 1999). This frequency range of peripheral encoding of sound covers the fundamental frequency of the male hum and several upper harmonics. Thus, unlike other organisms used to study the molecular mechanisms of auditory physiology such as mice, turtles, and zebrafish, the midshipman has simple vocalizations providing a behaviorally relevant acoustic context in which to study auditory encoding.

Immediately prior to the midshipman reproductive season, plasma levels of testosterone (males and females) and 17β-estradiol (females) peak and then decline through the breeding season (Sisneros et al., 2004b). Steroids modulate the vocal-motor system of males and females (Bass, 2007; Bass and Remage-Healey, 2008) and the auditory system of females (Sisneros et al., 2004a). Reproductive females collected from nests have saccular afferent neurons with robust temporal encoding (measured as vector strength of synchronization) of frequencies up to 340Hz at 130dB, while non-reproductive females best encode frequencies up to 100Hz (Sisneros and Bass, 2003). The increase in the encoding of the spectral content of the higher harmonics of male advertisement calls is proposed to aid females in localizing the

male call (and hence nest) because the second and third harmonics (~200 and 300Hz) contain close to as much energy (the second sometimes contains more) as the fundamental, but propagate farther through shallow water than the lower frequency fundamental (Fine and Lenhardt, 1983; Bass and Clark, 2003; Sisneros and Bass, 2003). Non-reproductive females show robust encoding of frequencies ≤100Hz. Treatment of non-reproductive females with either testosterone or estradiol implants for 23-37 days induces an auditory physiology phenotype like that of reproductive females, i.e., robust encoding of frequencies >100 Hz (Sisneros et al., 2004a). Steroid treatment replicates the approximately month long elevation of plasma concentrations of testosterone and estradiol in females prior to nesting (Sisneros et al., 2004b).

Like mammals, ERα mRNA is expressed in the auditory hair cell epithelium of the saccule of midshipman, as revealed by RT-PCR (Sisneros et al., 2004a) and *in situ* hybridization (Forlano et al., 2005). Aromatase (estrogen synthase) protein, which converts testosterone to estrogen, is expressed in the ganglion cells of the eighth nerve, as revealed by immunohistochemistry (Forlano et al., 2005). Together, these studies suggest that seasonal elevation of estrogen alone acts locally on the peripheral auditory system (for testosterone, following aromatization to estradiol) to increase temporal encoding of higher frequency sound.

A recent study measuring saccular microphonic potentials, the summed activity of hair cell populations in the sensory epithelium, has shown that changes in sound encoding occur at the level of the saccule, suggesting changes in hair cell tuning account for at least some of the seasonal changes observed at the afferent level (Sisneros, 2009). In Chapter 2, I further expand upon this study to characterize the

frequency dependence of seasonal plasticity in auditory hair cells in both male and female midshipman as well as describe a transitional auditory phenotype correlating with a transition between non-reproductive and reproductive states. Animals with an intermediate auditory phenotype had elevated circulating testosterone levels consistent with gonadal recrudescence as animals transition from a non-reproductive to reproductive state (Sisneros et al., 2004b). The results support the hypothesis that seasonal changes in circulating steroid levels trigger seasonal plasticity of saccular hair cells as has been demonstrated in saccular afferents (Sisneros et al., 2004a).

The morphology of the midshipman auditory system, specifically the accessibility of the saccule close to the surface of the skull (see Fig. 2.1C, Chapter 2), lends itself to further *in vivo* investigations of physiology using saccular potentials (Chapter 2) to characterize the role of BK channels in seasonal, reproductive state dependent shifts in frequency encoding (Cohen and Winn, 1967; Sisneros, 2007). The molecular mechanism underlying these changes is unknown. In this dissertation, I test the hypothesis that plasticity in frequency sensitivity of auditory hair cells is achieved via changes in the expression of BK channels in auditory hair cells.

Seasonal Auditory Plasticity

Chapter 2 presents an initial neurophysiological study in which I demonstrate two important attributes of the saccular auditory epithelium in midshipman fish. First, I show that saccular thresholds in males, profiled using auditory evoked potential recordings resembling cochlear microphonics (Furukawa and Ishii, 1967; Sisneros, 2007), display frequency-dependent seasonal plasticity (like females, Sisneros, 2009)

and that changing thresholds in males correlate with fluctuations in circulating steroid levels. Second, I show that non-reproductive males with elevated plasma testosterone levels have a saccular phenotype intermediate between non-reproductive and reproductive conditions, consistent with the dependency of gradual transitions in auditory phenotype in the wild on pre-nesting rises in plasma steroid levels. Next, after cloning the *slo1* genes in midshipman as described in Chapter 3, I demonstrate in Chapter 4 seasonal changes in expression of at least one of the *slo1* genes in the auditory saccular epithelium of midshipman, consistent with elevated *slo1* and BK channel expression facilitating higher resonance frequencies during the reproductive season. Also shown in Chapter 4, by reducing the number of available BK channels via pharmacological manipulation I was able to induce frequency dependent changes in auditory hair cell physiology. These changes to reproductive animals resulted in auditory hair cell evoked responses characteristic of non-reproductive individuals.

BK Channel Genes

As explained in detail in Chapter 3, the *slo1* gene appears to be one of many ion channel genes duplicated as part of a whole genome duplication in the line of ray-finned fish yielding the teleost lineage. Recent studies report duplication of the sodium channel gene Nav1.4 (Zakon et al., 2006), several voltage-gated calcium channel alpha1-subunit genes (Wong et al., 2006), and most of the genes in the KCNA/Kv1/shaker-related voltage-gated potassium channel family (Few and Zakon, 2007; Hoegg and Meyer, 2007). The high rate of retention of duplicated ion channel genes in teleosts compared to baseline retention rate of ~20-50% (Lynch and Force,

2000) suggests that paralogues have undergone positive selection by divergence of function either through developing novel functions or through division of subfunctions between duplicated genes. Either case argues for the comparative study of expression and regulation of ion channels produced by teleost genes that are co-orthologues of a single tetrapod gene. In Chapter 3, I characterize the duplication of teleost *slo1* genes across a variety of teleosts and identify tissue specific subfunctionalization of expression as a mechanism for retention of duplicate *slo1* genes in midshipman.

BK Channels and Electrical Tuning

Electrical tuning of hair cells following mechanoelectrical transduction of sound is the primary mode of frequency tuning of hair cells (Fettiplace and Fuchs, 1999). Electrical tuning is the oscillation of membrane potentials in response to pulsed current injections similar to those elicited by brief acoustic stimulation of the cell (Fettiplace and Fuchs, 1999). This resonance is the result of activation of voltage-gated calcium currents depolarizing the cell followed by activation of BK channel currents to repolarize the cell. The decay rate of oscillations is determined by the number and type of BK channels as well as the number of voltage-gated calcium channels (Fettiplace and Fuchs, 1999). In turtle (*Trachemys scipta elegans*), the distribution of *Slo1* gene splice variants encoding the BK channel α-subunit, as well as a β-subunit along the basilar membrane (cochlea homologue), produce a gradient of cells with different BK channel currents that can explain the frequency range of turtle hearing between 30 and 600Hz (Jones et al., 1998; Jones et al., 1999b; Jones et al., 1999a). The frequency range at which BK channel diversity accounts for electrical tuning in turtles and other

vertebrates (Fettiplace and Fuchs, 1999) encompasses the frequency range of midshipman hearing. BK channels play an analogous role in the membrane oscillations of saccular hair cells in goldfish (Sugihara and Furukawa, 1989; Sugihara, 1994), and have been identified as a major outward current in saccular hair cells of toadfish (*Opsanus tau*) (Steinacker and Romero, 1991), a species in the same family (Batrachoididae) as midshipman (Nelson, 2006). The rapid kinetics of both activation and deactivation of BK currents are necessary for electrical resonance in toadfish saccular hair cells (Steinacker and Romero, 1992). Despite the fact that mammals use mechanical tuning instead of electrical tuning (Fettiplace and Fuchs, 1999), BK currents are a large contributor to the outward currents in mammalian inner (Skinner et al., 2003) and outer (Wersinger et al., 2010) hair cells. Several studies using knockouts of either or both BK α and β subunits have shown that the pore forming BK α-subunit, and not the β1 or β4 subunits, is necessary for normal mouse cochlear function (Ruttiger et al., 2004; Oliver et al., 2006; Pyott et al., 2007). Unlike tetrapods such as turtles, expression of BK channels had not been previously examined at the molecular level in fish at the time the experiments in this dissertation were commenced.

Steroids, BK Channels and Auditory Plasticity

Steroid hormones are a regulator of *Slo1* expression and alternative splicing. The approximately month long time course of estradiol treatment necessary to induce a reproductive auditory phenotype in a non-reproductive female midshipman argues for a genomic mechanism. Glucocorticoids and testosterone regulate expression of *Slo1* transcripts containing the STREX exon in, respectively, adrenal chromaffin cells (Xie

and McCobb, 1998; Lai and McCobb, 2002, 2006) and the pituitary (Mahmoud and McCobb, 2004). STREX transcripts decrease throughout pregnancy in rat myometrium, an effect replicated by estrogen treatment and blocked by ER antagonists (Zhu et al., 2005). Additional *Slo1* splice variants that modulate calcium and voltage sensitivity of the BK channel are upregulated by 17β-estradiol in mouse myometrium (Holdiman et al., 2002). Estrogen may also regulate *Slo1* gene transcription in general as indicated by the presence of multiple estrogen-response elements in some of the promoters of mouse *Slo1* (Kundu et al., 2007). Combined with its importance in electrical resonance of auditory hair cells and regulation of its expression by estrogen, the BK channel is a good target for investigating molecular mechanisms of seasonal estrogen-dependent auditory plasticity. As discussed in the conclusion of this dissertation, Chapter 5, I project forward as I attempt to extend the information gathered from studies of auditory hair cell plasticity in midshipman fish to the broader question of auditory plasticity in vertebrates, with specific focus of ontogenetic plasticity under the influence of steroid hormones in mammals including humans.

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CHAPTER 2

SEASONAL PLASTICITY OF AUDITORY HAIR CELL FREQUENCY SENSITIVITY CORRELATES WITH PLASMA STEROID LEVELS IN VOCAL FISH

Abstract

Vertebrates displaying seasonal shifts in reproductive behavior offer the opportunity to investigate bidirectional plasticity in sensory function. The midshipman teleost fish exhibits steroid-dependent plasticity in frequency encoding by eighth nerve auditory afferents. Here, evoked potentials were recorded *in vivo* from the saccule, the main auditory division of the inner ear of most teleosts, to test the hypothesis that males and females exhibit seasonal changes in hair cell physiology in relation to seasonal changes in plasma levels of steroids. Thresholds across the predominant frequency range of natural vocalizations were significantly less in both sexes in reproductive compared to non-reproductive conditions, with differences greatest at frequencies corresponding to call upper harmonics. A subset of non-reproductive males exhibiting an intermediate saccular phenotype had elevated testosterone levels, supporting the hypothesis that rising steroid levels induce non-reproductive to reproductive transitions in saccular physiology. We propose that elevated steroids act via long-term (days to weeks) signaling pathways to upregulate ion channel expression generating higher resonant frequencies characteristic of non-mammalian auditory hair cells, thereby lowering acoustic thresholds.

*In press (without appendix) as Rohmann KN and AH Bass. *Journal of Experimental Biology*.

Introduction

Vertebrate sensory systems undergo ontogenetic changes that can have a profound impact on an organism's ability to hear and communicate acoustically. Mammals show marked decline in auditory sensitivity with age, particularly at higher frequencies within their respective audible ranges (for review see Ohlemiller, 2006). Songbirds show remarkably stable baseline auditory thresholds throughout ontogeny (Langemann et al., 1999) with species-specific seasonal changes (Lucas et al., 2002; Lucas et al., 2007), while studies in fishes have yielded conflicting results (Popper, 1971; Kenyon, 1996; Iwashita et al., 1999; Higgs et al., 2002; Higgs et al., 2003; Sisneros and Bass, 2005). Here, we investigate peripheral auditory plasticity in a teleost fish, the plainfin midshipman (*Porichthys notatus* Girard 1854, family Batrachoididae), that shows seasonal, reproductive state-dependent plasticity in the ability to encode the upper harmonics of vocalizations (Figure 2.1A, B). As females shift from a non-reproductive to a reproductive state, they exhibit a steroid-dependent improvement in frequency encoding by eighth nerve afferents to the saccule (Sisneros and Bass, 2003; Sisneros et al., 2004a), the main auditory division of the inner ear in midshipman and most teleosts (Figure 2.1C insert, Popper and Fay, 1993; McKibben and Bass, 1999). We tested the hypothesis that this plasticity is not sex-dependent, with males also exhibiting concurrent shifts in plasma steroid levels and auditory encoding as reflected in frequency sensitivity of the saccule's hair cell epithelium.

Much of the work on steroid modulation of hearing throughout ontogeny focuses on changes over the entire life span of an animal. For example, human females have enhanced auditory brainstem response performance over males

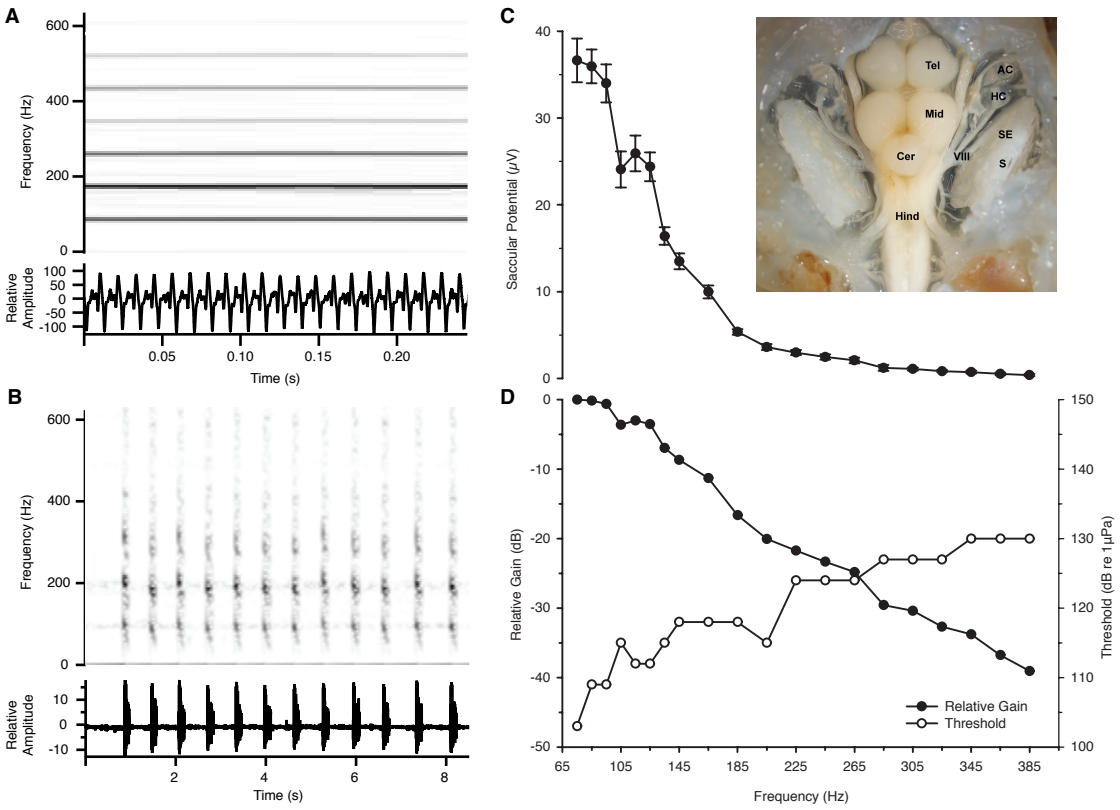


Figure 2.1

Plainfin midshipman (*Porichthys notatus*) vocalizations and saccular microphonics. Type I male midshipman vocalizations including a courtship "hum" (A), and antagonistic "grunt train" (B) with spectrograms plotted above the waveform for each vocalization. Note the expanded time base in (A) to show the fine structure of the hum which lasts for minutes to hours while the complete duration of a series of grunts (B) is visible. (C) Representative iso-level response recorded from saccule in response to single tones at 130 dB. Saccular potentials are plotted as mean ± SD with SD bars obscured by many of the points. Inset: dorsal view of midshipman brain and inner ear. AC, anterior canal ampulla; Cer, cerebellum; HC, horizontal canal ampulla; Hind, hindbrain; Mid, midbrain; S, saccule; SE, saccular epithelium; Tel, telencephalon; VIII, eighth cranial nerve. (D) Representative examples of both an individual iso-level response curve of evoked saccular potentials and an individual auditory threshold tuning curve obtained from the set of recordings from a reproductive male of which (C) was a single iso-intensity response. Auditory threshold was defined for each frequency as the stimulus intensity in dB (re 1 μPa) that evoked a saccular potential ! 2 SD above background noise measurements.

(Jerger and Hall, 1980; Don et al., 1993), while both sexes show marked deficits with increasing age (Jerger and Hall, 1980) that can be slowed with estrogen treatment in postmenopausal women (Kilicdag et al., 2004). The midshipman fish studies investigate yearly events as animals transition between non-reproductive and reproductive states (Sisneros and Bass, 2003; Sisneros et al., 2004a; Sisneros, 2009). So far, this work has focused on females because early behavioral studies showed females exhibiting robust and consistent positive phonotaxis to playbacks mimicking male advertisement "hums" (Figure 2.1A) that females use to localize nesting males (Brantley and Bass, 1994; McKibben and Bass, 1998, 2001). In these studies, males also responded positively to hum playbacks (McKibben and Bass, 1998). In addition, like females, males exhibit increased plasma steroid levels coinciding with gonadal recrudecense prior to onset of reproduction (Brantley et al., 1993a; Knapp et al., 1999; Sisneros et al., 2004b). Lastly, the peripheral auditory system of both sexes express androgen and estrogen receptor mRNAs and the steroidogenic enzyme aromatase/ estrogen synthase (Forlano et al., 2001; Forlano et al., 2005; Forlano et al., 2010). Together, these findings strongly suggested that, like females, peripheral auditory physiology among male midshipman would exhibit seasonal plasticity that parallels changing gonadal status and levels of circulating steroids.

Midshipman fish have two male reproductive morphs. Type I, territorial males build and defend nests, and acoustically court females with hums (Figure 2.1A). Type II males sneak/satellite-spawn and neither build nests nor engage in acoustic courtship (Brantley and Bass, 1994). Both male morphs and females produce agonistic grunts (e.g., Figure 2.1B). Our primary goal was to test the hypothesis that type I males

exhibit seasonal auditory plasticity at the hair cell level comparable to that recently shown for females (Sisneros, 2009) and that this seasonality correlates with reproductive state and levels of circulating steroid hormones. Types I males were the focus because they show seasonal plasticity in vocal physiology (unknown for type II's) and wild-caught populations of both non-reproductive and reproductive individuals are readily available (Rubow and Bass, 2009). Our findings are significant in several regards. First, saccular hair cell physiology in males, profiled using auditory evoked potential recordings resembling cochlear microphonics (Furukawa and Ishii, 1967; Sisneros, 2007), showed that seasonal changes in saccular thresholds correlated with fluctuations in circulating steroid levels. Second, we fortuitously collected a subset of non-reproductive males with elevated plasma testosterone levels and a saccular phenotype intermediate between non-reproductive and reproductive conditions. These results were consistent with the dependency of gradual transitions in auditory phenotype in the wild on pre-nesting rises in plasma steroid levels. Additional analyses investigated if saccular physiology and steroid levels vary as a function of morphometric measures (standard length, relative gonad weight), time in captivity, and time of day. Unlike vocal motor excitability (Rubow and Bass, 2009), we report no measurable diel shifts in saccular hair cell physiology among reproductive males, showing the restriction of this form of acoustic plasticity to comparatively longer-term seasonal patterns.

Materials and Methods

Animals

This study included 29 type I male and 19 female plainfin midshipman collected during the 2009 non-reproductive (February) and reproductive (May-August) seasons as well as the non-reproductive season of 2010 (December 2009). Reproductive animals were hand collected from nests in the rocky intertidal zone in Tomales Bay, California. Non-reproductive animals were collected by otter trawl off the coast of California (December 2009) and Washington (February 2009). All animals were shipped to Cornell University and housed in saltwater aquaria at ~16˚C and fed a diet of goldfish. During the reproductive season, animals were housed under a 14 light (L) : 10 dark (D) light cycle with lights out at 1800 h Eastern Daylight Time while non-reproductive animals were housed under a 10L : 14D cycle with lights out at 1700 h Eastern Standard time (EST) (Rubow and Bass, 2009). All experiments were conducted during the subjective day except for a subset which were conducted after 18:00 h EST during the subjective night to test the hypothesis that peripheral auditory plasticity displays a daily cycle as found in the vocal motor system (Rubow and Bass, 2009).

Type I males caught any time of year are distinguishable from type II males on the basis of multiple somatic traits (Brantley et al., 1993a; Brantley and Bass, 1994; Bass, 1996; Sisneros et al., 2004b). Reproductive state of the animals was confirmed by visual inspection of the gonads (e.g., presence of mature ovarian follicles in gravid reproductive females) as well as by measurement of the gonadosomatic index (GSI, 100 X ratio of gonad mass to body mass – gonad mass) (Tomkins and Simmons, 2002). Additionally, type I male vocal muscle was visually inspected as its enlargement is a prominent secondary sex characteristic induced by elevated plasma

androgen levels prior to and during the reproductive season (Brantley et al., 1993b). All methods were approved by the Institutional Animal Care and Use Committee at Cornell University.

Stimulus Generation

Methods were adapted from those previously published (Sisneros, 2007, 2009). Sound stimuli were presented via underwater speaker (UW-30, Telex Communications, Burnsville, MN) positioned 10 cm below the fish's head during recordings or hydrophone during calibrations. The speaker was partially embedded in the gravel substrate at the bottom of a 30 cm diameter by 24 cm high Nalgene tank, adopted from methods similar to those used in previous auditory studies of midshipman and other fishes (Fay, 1990; McKibben and Bass, 1999; Bass and McKibben, 2003). Sine wave acoustic stimuli were generated by the reference signal of an SR830 lock-in amplifier (SR830, Stanford Research Systems, Sunnyvale, CA) fed through an audio amplifier that powered the underwater speaker. Custom written Matlab software was used to control stimulus generation through the lock-in amplifier. Stimuli consisted of 500 ms tones played at 1.5 s intervals repeated eight times. Tones were presented in 10 Hz (75-145 Hz) and 20 Hz increments (165-385 Hz) in random order. For iso-level responses each frequency was played separately at 130 dB re 1 μPa, the approximate sound pressure level of the fundamental frequency of the male courtship hum (Figure 2.1A) measured at a distance (10 cm) comparable to that between the fish and the stimulus source in our experimental setup (Bass and Clark, 2003). Stimuli were

presented at 3 dB increments above and below 130 dB from 100-151 dB re 1 μPa to construct threshold tuning curves (see below).

A Bruel and Kjaer 8103 mini-hydrophone (Norcross, GA) was positioned 10 cm above the underwater speaker in the position occupied by the head of the fish during physiological recordings in order to calibrate the stimuli. Relative sound measures were fed from the hydrophone to a SR780 signal analyzer (Stanford Research Systems, Sunnyvale, CA) for FFT analysis and to an oscilloscope for peak-to-peak voltage measurements. Stimulus amplitudes were then adjusted in Matlab so relative sound pressures across all frequencies (75-385 Hz) were within a ± 1 dB window. While all calibrations and data are reported as measures of pressure, previous studies have shown that the midshipman peripheral auditory system is primarily sensitive to particle motion and not pressure (Weeg et al., 2002), like many other fishes which lack specialized adaptations for pressure detection (Popper and Fay, 1993). Despite their sensitivity to particle motion, midshipman auditory afferents respond similarly to both iso-pressure stimuli from sound sources such as those used in the present study and vertical acceleration (Weeg et al., 2002). As has been discussed extensively (McKibben and Bass, 1999; Weeg et al., 2002; Sisneros and Bass, 2003; Sisneros, 2009), sound pressure provides one way to measure stimulus intensity; in the case of this study and others that focus on comparisons of peripheral auditory function between populations using fixed stimuli and recording techniques (Sisneros and Bass, 2003; Sisneros et al., 2004a; Sisneros, 2009) absolute measures of stimulus intensity in terms of particle motion are not necessary to make quantified comparisons of relative differences between groups.

Saccular Potential Recordings

Recording methods were adapted from those used previously to record saccular potentials in midshipman (Sisneros, 2007, 2009). Briefly, both saccules were exposed by dorsal craniotomy following anesthetization via immersion in 0.025 % ethyl p-aminobenzoate (benzocaine, Sigma-Aldrich, St. Louis, MO, USA) in saltwater followed by intramuscular injection of pancuronium bromide (~ 0.5 mg / kg, Tocris, Ellisville, MO, USA) for immobilization and subcutaneous injection of 0.25 % bupivacaine (~ 1mg / kg, Hopsira, Inc., Lake Forest, IL, USA) with epinephrine (0.1 mg / ml, International Medication Systems, El Monte, CA, USA) for analgesia. A denture cream dam ~ 2 cm high was constructed around the exposed cranium so the cranium could be lowed below the water line in the experimental tank. The fish was positioned 10 cm above the stimulus speaker in the chamber described above (see Figure 2 of McKibben and Bass, 1999 for diagram of experimental setup). The experimental tank was placed on a vibration isolation table within an acoustic isolation chamber (Industrial Acoustics, Bronx, NY, USA) while all hardware for stimulus generation and recording of potentials was positioned outside the acoustic chamber. Fish were perfused through the mouth with recirculated chilled (15-16˚C) saltwater providing flow over the gills for respiration.

A glass recording microelectrode (1-7 MΩ) containing 3 M KCl was inserted into the saccular endolymph approximately 2-4 mm from the sensory epithelium in one of several recording positions along the rostral-caudal axis of the epithelium (rostral, middle, caudal) in either the left of right saccule. Saccular potentials were

amplified (Model 5A, Getting Instruments, San Diego, CA, USA), band pass filtered and further amplified (SR650, Stanford Research Systems, Sunnyvale, CA, USA), and fed into the lock-in amplifier (mentioned above) for analog to digital conversion and signal processing and stored on a PC computer using custom written Matlab software (Figure 2.1C). The lock-in amplifier was configured with a time constant of 100 msec and bandwidth of 12 dB. As discussed in more detail below, saccular potentials are evoked at twice the stimulus frequency in fishes including midshipman (Cohen and Winn, 1967; Furukawa and Ishii, 1967; Hama, 1969; Fay and Popper, 1974; Sisneros, 2007, 2009). The lock-in amplifier uses the stimulus frequency as a reference to output a DC signal with voltage proportional to the signal component at a multiple of the stimulus frequency. Except where noted below, the lock-in amplifier was set to generate outputs at twice the reference (stimulus) frequency in order to isolate evoked responses of saccular hair cells. Iso-level responses were normalized in order to control for variability in distance of electrode from sensory epithelium with all responses expressed relative to 0 dB assigned to the maximum evoked potential at the peak frequency sensitivity (Figure 2.1C, D).

Threshold tuning curves were constructed using methods adapted from those previously published (Figure 2.1D, Sisneros, 2007). Background noise measurements were made at the beginning of each set of recordings with no sound stimulus and were used to calculate a response threshold for each frequency. Threshold was set as the minimum amplitude acoustic stimulus needed to elicit a response with amplitude greater than 2 standard deviations above the mean background noise measurement. Threshold tuning curves were constructed by comparing mean responses (average of

responses recorded during the eight repetitions of the 500 ms acoustic stimulus at a given frequency) recorded in response to sounds in 3 dB increments above and below the 130 dB reference recording, ranging from 100-151 dB re 1 μPa.

Hormone Assays

Plasma testosterone concentration was measured in duplicate 50 μL aliquots using a solid phase 125I radioimmunoassay (RIA) kit (Siemens, Los Angeles, CA). Cross-reaction of the testosterone antibody with 5α-dihydrotestosterone was 3.4%, with 11-keto-testosterone was 16 %, and the minimum detectable limit of the assay was 0.04 ng / ml according to the manufacturer. A pooled sample of plasma from four reproductive type I males was used to validate the testosterone assay. The intra- and inter-assay coefficients of variation were 0.03 (n = 5 replicates) and 0.11 (n = 4 replicates), respectively.

Testis Histology

Whole testis from winter 2010 males was immersion fixed in 0.4 % paraformaldehyde in 0.1 M phosphate buffer overnight at 4˚C, washed several times in 0.1 M phosphate buffer, and cryoprotected in 30% sucrose overnight at 4˚C before sectioning at 30 μm in a cryostat. Sections were dried and mounted onto superfrost slides, stained in cresyl violet, dehydrated in an ethanol series, cleared with xylene, and coverslipped. The presence of mature and immature sperm was then visualized under a light microscope.

Statistical Analysis

All statistical analyses were performed in JMP 8 (S.A.S. Institute Inc., Cary, NC). The effect of reproductive state on thresholds and iso-level responses was determined by a multilevel, repeated measures statistical model with reproductive state as the between-subject factor. Iso-level response or threshold was a response variable of each stimulus frequency. All responses across frequencies for a given recording position were nested within a single trial characterized by recording position (rostral, middle, caudal) and side (left, right) which were, in turn, nested within an individual fish which was characterized by a reproductive state. This model also allowed us to test whether recording position or side had an effect on recordings. Additionally, morphometric data on individual animals including standard length, GSI and plasma testosterone levels were added to determine if they explained any additional variance between groups not already accounted for by reproductive condition. For analyses with more than two groups (e.g., comparison of males from three seasons) Tukey-Kramer HSD post-hoc tests were used to test for differences between pairs of groups. The same model was used to test the effect of time of day and duration in captivity on thresholds and iso-level responses, replacing reproductive state with each of the aforementioned as the character state assigned to each fish within the model. The effect of season on plasma testosterone levels was determined by ANOVA with Tukey-Kramer HSD post-hoc comparisons between individual groups. The remaining analyses of relationships between GSI, SL, time in captivity, and plasma testosterone levels were performed using ANOVA on simple linear regressions.

Results

Methodological Verification: Frequency Doubling of Evoked Saccular Potentials

As reported previously, in midshipman (Cohen and Winn, 1967; Sisneros, 2007, 2009) and other fishes (Furukawa and Ishii, 1967; Hama, 1969; Fay and Popper, 1974), evoked saccular potentials are usually recorded at twice the stimulus frequency. This has been explained by the presence of two populations of oppositely oriented hair cells in fishes whose potentials sum non-linearly (Furukawa and Ishii, 1967; Hama, 1969; Fay, 1974; Fay and Popper, 1974). However, some of these same studies reported locations where the evoked response was primarily at the stimulus frequency and not the second harmonic (Cohen and Winn, 1967; Furukawa and Ishii, 1967; Furukawa et al., 1972; Fay, 1974). Previous studies in midshipman only measured responses at both the fundamental and second harmonic at a single iso-intensity (130dB re 1 μPa, Figure 2.1C, D, Sisneros, 2007, 2009). We sought to rigorously test whether saccular responses at the fundamental and second harmonic were different across stimulus intensities, in part, because of reports in mosquitoes in which oppositely oriented mechanotransducers in the antennae produce a strikingly similar frequency doubling effect as seen in fishes but only at lower frequencies (Tischner, 1953; Wishart et al., 1962; Arthur et al., 2010). Similarly, Sisneros (2007) showed previously that at 130 dB re 1 μPa there appeared to be a frequency (~ 145 Hz) above which the response at the fundamental was greater than the response at twice the stimulus frequency. Unfortunately, the responses at the frequencies in question were at or below threshold at 130 dB re 1 μPa as determined by the electrical noise of the rig. By examining responses over a wider range of stimulus intensities we sought to more rigorously

examine whether frequency doubling is present in the saccular responses of midshipman across all stimulus frequencies.

Unlike recording saccular potentials at twice the stimulus frequency, recordings made at the fundamental using the lock-in amplifier (see Materials and Methods) were subject to additional sources of electrical noise including, but not limited to, physical vibration of the recording electrode and electromagnetic fields induced by the underwater speaker. Because it was not possible to physically isolate the underwater speaker from the recording electrode and the placement of electromagnetic shielding between the underwater speaker and the animal could potentially disturb the underwater sound field, we were unable to completely eliminate these sources of noise. As such, we had to use different criteria for determining the threshold for saccular responses. Background noise recordings were made during acoustic playback after each tuning curve with the electrode placed in the saltwater chamber in the position it had been while recording from inside the saccule. Auditory threshold at each stimulus frequency was defined as the lowest stimulus intensity that evoked a saccular potential greater than the noise reading. This was a rather conservative estimate of threshold as it assumed that the noise and evoked saccular responses summed linearly without any phase cancellation between the two signals. Comparing the threshold tuning curves recorded at the fundamental and twice the stimulus frequency revealed no notable differences between the two recordings (n = 2, 2) with thresholds varying by only approximately 3 dB at any given frequency. Such a minor difference between tuning curves was equivalent to one step in sound intensity and was within the range of variability observed between threshold tuning curves

collected at twice the stimulus frequency. While we were limited by the noise present in recordings made at the stimulus frequency which were not present in recordings at twice the stimulus frequency, we were reasonably certain that by recording only the responses at twice the stimulus frequency we were not overlooking potentials evoked at the stimulus frequency as both recording methods yielded similar results. We chose to continue recording saccular responses at twice the stimulus frequency and to use the criteria for determining threshold of responses used previously (Sisneros, 2007, 2009) for all other results reported in this study.

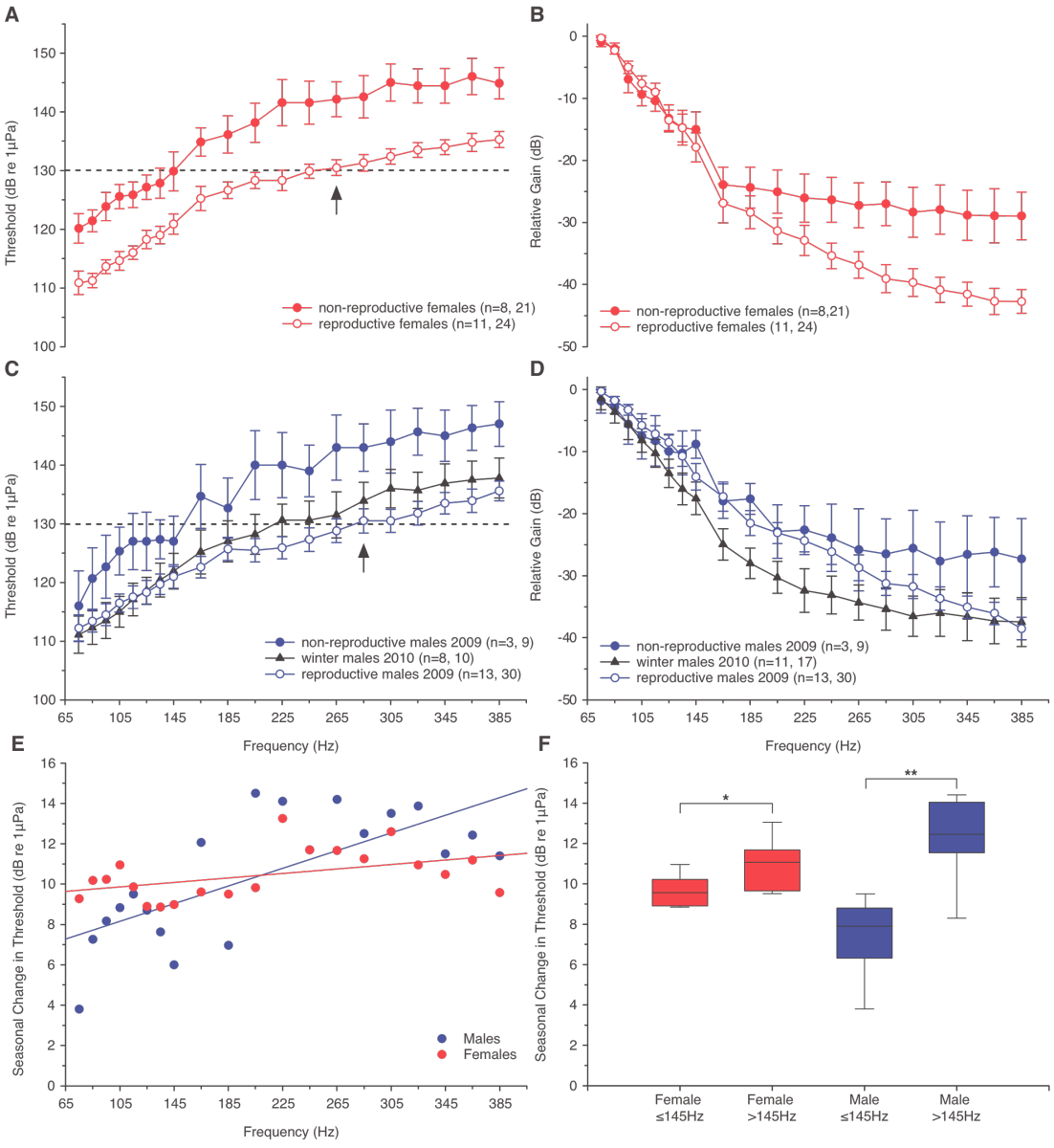
Seasonal Plasticity of Saccular Sensitivity in Females

Given the potential for differences in recording setups and hence acoustics, we first replicated prior studies of seasonal saccular plasticity in female midshipman (Sisneros, 2009) to both determine consistency of the plasticity and test the null hypothesis that, for the same wild-caught population of fish, males would not exhibit seasonal changes in auditory phenotype like females. Evoked saccular potentials were recorded from 19 adult midshipman females: 8 non-reproductive females with a size range of 9.7-12.3 cm (mean standard length, SL ± s.d. = 11.2 ± 0.9 cm) collected in February 2009 and housed in captivity for 14-30 days; 11 reproductive females, 11.6-15.6 cm (mean SL = 13.1 ± 1.1 cm), collected May – August 2009 and captive for 3-15 days. Auditory thresholds were significantly lower in reproductive than non-reproductive females (multi-level repeated-measures model; between-subject factor reproductive state $p < 0.0001$, Figure 2.2A) with a significant interaction of frequency and reproductive state ($p = 0.04$, see Figure 2.2E). Because there was no significant effect of either side (i.e.,

Figure 2.2

Saccular thresholds show frequency-dependent seasonal plasticity. (A, C) Auditory threshold tuning curves for non-reproductive and reproductive female (A) and male (C) midshipman based on evoked saccular potentials. Vertical arrows mark frequencies above which threshold is greater than the 130 dB stimulus level used in iso-level responses shown in (B) and (D). (B, D) Evoked potentials recorded from the saccule of non-reproductive and reproductive female (B) and male (D) midshipman in response to iso-level tones of 130 dB (re 1 μPa). Data were normalized with a relative gain value of 0 dB assigned to the peak response for each recording with all other responses expressed as relative dB re the frequency with the largest response. (A-D) data are plotted as means ± 95 % confidence limit (CL) with the number of animals and records indicated in parentheses. (E) Change in mean threshold between reproductive and non-reproductive seasons in female (see A) and male (see C) midshipman as a function of frequency. (F) Box plot of change in mean threshold between reproductive and non-reproductive seasons in female and male midshipman for each frequency ≤ or > 145Hz. * denotes a statistically significant ($p < 0.05$) difference between groups. ** denotes a statistically significant ($p < 0.0001$) difference between groups.

Figure 2.2 (continued)



left or right saccule; $p = 0.48$) or electrode recording position (rostral, middle, caudal; $p = 0.92$) on threshold, all recordings were pooled together for analysis as summarized in Figure 2.2A. Additionally, there was no effect of standard length on threshold ($p = 0.34$).

Similarly, iso-level responses of evoked saccular potentials revealed reproductive females had a greater relative gain than non-reproductives (multi-level repeated-measures model: between-subject factor reproductive state $p = 0.0006$, Figure 2.2B) with a significant interaction of frequency and reproductive state ($p < 0.0001$). Range of relative gain (responses normalized relative to 0 dB assigned to the maximum evoked potential at the peak frequency sensitivity) showed similar differences between reproductive (43 dB) and non-reproductive (29 dB) animals as reported previously (44 dB and 31 dB, respectively, Sisneros, 2009). As with threshold tuning curves, there was no significant effect of either side ($p = 0.53$) or electrode recording position ($p = 0.61$) so all recordings were pooled for analysis and summarized in Figure 2.2B.

These results demonstrated that seasonal plasticity of the female saccular phenotype is highly stable; animals collected and tested during 2006 and 2007 (Sisneros, 2009) exhibited similar saccular responses as those in 2009 (current study). These results also confirmed the consistency of recording techniques developed by Sisneros (2007, 2009) for midshipman and Furukawa for goldfish (Furukawa and Ishii, 1967), and that transporting and maintaining animals at Cornell University did not have any noticeable effect on saccular physiology. Thus, we were confident in making comparisons to possible seasonal changes in male auditory phenotypes.

Seasonal Plasticity of Saccular Sensitivity in Males

Evoked saccular potentials were recorded from 16 type I male midshipman: 3 non-reproductive males with a size range of 10.4-10.8 cm (mean SL = 10.6 ± 0.2 cm) collected during February 2009; 13 reproductive males, 12.2-17.7 cm (mean SL = 14.4 ± 1.9 cm) collected during May - August 2009. There were significant threshold differences between non-reproductive and reproductive males collected in 2009 (multi-level repeated-measures model: between-subject factor reproductive state $p = 0.0069$, Figure 2.2C) with a significant interaction of frequency and reproductive state ($p < 0.0001$, see Figure 2.2E). Examination of threshold tuning curves (Figure 2.2C) suggested an increase in seasonal differences at frequencies > 145Hz. Seasonal differences in mean threshold were significantly greater at frequencies > 145Hz than ≤ 145Hz in both males ($p < 0.0001$, ANOVA) and females ($p = 0.02$, ANOVA) (Figure 2.2F). Generally, reproductive males had lower thresholds than non-reproductive animals (Figure 2.2C). Recordings were pooled between sides ($p = 0.10$) and recording position ($p = 0.84$). There was no significant effect of standard length on thresholds ($p = 0.19$). There were also no statistical differences in thresholds between sexes in either reproductive (Tukey-Kramer HSD: $p = 0.99$) or non-reproductive (Tukey-Kramer HSD: $p = 0.95$) animals.

Unlike females, there was no significant difference in iso-level responses of evoked saccular potentials between reproductive and non-reproductive males collected in 2009 (multi-level repeated-measures model: between-subject factor reproductive state $p = 0.31$, Figure 2.2D). However, males followed the same trend as females with

non-reproductive animals having a smaller range of relative gain of responses (27.3 dB) in the saccule compared to reproductive animals (38.5 dB). Recordings were pooled between sides ($p = 0.47$) and recording position ($p = 0.53$). The discussion provides a detailed analysis of discrepancies between threshold and iso-level response data.

While type I males and females showed similar seasonal plasticity of saccular thresholds between reproductive and non-reproductive seasons in 2009, males collected during the 2010 non-reproductive season (December 2009) showed an intermediate phenotype. Evoked saccular potentials were recorded from 11 type I males with a size range of 13.0-18.8 cm (mean SL = 15.1 ± 1.9 cm). There was an overall effect of collection period on thresholds amongst the three collecting seasons (multi-level repeated-measures model: between-subject factor collecting season $p = 0.01$, Figure 2.2C) with a significant interaction of frequency with collecting season ($p < 0.0001$). Thresholds were not significantly different between 2010 animals and either non-reproductive (Tukey-Kramer HSD: $p = 0.07$) or reproductive males (Tukey-Kramer HSD: $p = 0.60$) from 2009 (Figure 2.2C). Recordings were pooled between sides ($p = 0.13$) and recording position ($p = 0.66$). Iso-level responses of evoked saccular potentials were not significantly different between these three groups (multi-level repeated-measures model: between-subject factor collecting season $p = 0.12$, Figure 2.2D).

Unlike previous analyses, there was a significant effect of standard length on threshold ($p = 0.03$). Further analyses revealed an absence of any significant interaction between standard length and collecting season ($p = 0.74$) resulting in loss

of significance in the effect of standard length on threshold ($p = 0.73$). This reflected the overall size difference between non-reproductive /winter (smaller) and reproductive/summer animals, but indicated the effect of collecting season on thresholds is not due to differences in standard length between seasons.

Together, the results demonstrated that males, like females, display frequency-dependent seasonal plasticity in saccular thresholds.

Daily Rhythms in Saccular Physiology

Type I males show diel and seasonal shifts in vocal motor excitability: compared to non-reproductives tested any time of day and reproductives tested during the day, nocturnal reproductive type I males show both a marked increase in duration of, and decrease in threshold to elicit, the vocal motor volley that directly sets natural call duration and frequency (Rubow and Bass, 2009). Because the vocal system has direct input to central auditory circuitry (Bass et al., 1994; Weeg et al., 2005; Chagnaud et al., 2009), a subset of reproductive type I males ($n = 4$ animals, 9 tuning curves) were tested during the subjective night. Recordings of evoked saccular potentials from night animals were not markedly different from those recordings made during the day. Thresholds recorded at night (not shown) were not significantly different from those recorded during the day (multi-level repeated-measures model: between-subject factor time of day $p = 0.36$) and were within the range of variability seen between individuals recorded during the day and were thus included in the larger data set.

Plasma Testosterone Levels

A subset of type I males collected during the non-reproductive /winter season of 2010 showed large, vascularized vocal muscles and/or enlarged testes, traits associated with transitioning from a non-reproductive to reproductive state (Brantley and Bass, 1994; Bass, 1996; Sisneros et al., 2004b). Sections through testes of a subset of winter 2010 males showed the presence of mature and immature sperm (not shown, see Sisneros et al., 2004b), consistent with earlier studies of midshipman showing an increase in the number of mature sperm during the pre-nesting period leading up to the reproductive season. Male testes collected during the winter 2009 were not sectioned as there was no indication of transitioning from non-reproductive to reproductive states as described above for winter 2010 animals (i.e., gonads and vocal muscle were small and poorly vascularized). Our previous study showed that the testes of non-reproductive males contain no mature sperm (Sisneros et al., 2004b).

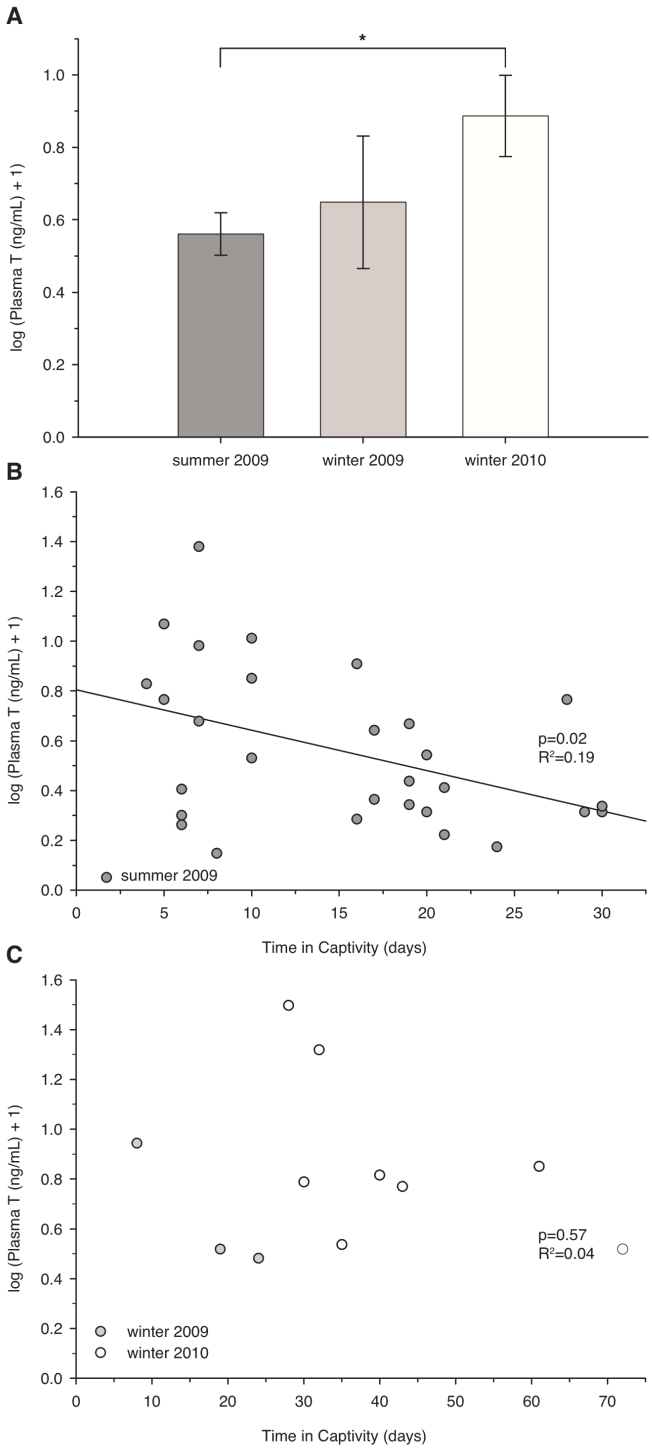
Circulating steroids, including testosterone, increase in both male and female midshipman during this transition (Sisneros et al., 2004b). Plasma testosterone levels were measured using RIA to determine whether winter 2010 animals were undergoing this transition between non-reproductive and reproductive states. Samples were collected following saccular recordings from 40 type I males across three seasons (n = 29 reproductive/ summer 2009, n = 3 non-reproductive /winter 2009, and n = 8 non-reproductive /winter 2010). Due to limited plasma volume, several samples were tested in singleton instead of duplicate (n = 5 summer 2009, n = 3 winter 2009, n = 3 winter 2010). Plasma testosterone levels were log transformed for statistical analyses because of unequal variance between populations due at least in part to the relatively small sample sizes for winter animals. Log transformed plasma testosterone levels

were significantly different between the three groups of males (ANOVA $p < 0.05$, Figure 2.3A) with non-reproductive/winter 2010 animals having elevated levels compared to 2009 reproductive/summer animals (Tukey-Kramer HSD $p = 0.04$). Thus, unlike non-reproductive/winter 2009 animals, non-reproductive/winter 2010 males had apparently begun to transition to a reproductive state with gonadal recrudescence and increased testosterone synthesis consistent with the prevalence of mature sperm and enlarged/vascularized vocal muscle in the winter 2010 males (see above). These differences were the basis for separating the physiological data collected from winter males into two populations based on the year in which they were collected. The threshold data for all three study populations (Figure 2.2C) revealed that log plasma testosterone levels had no additional significant effect on threshold ($p = 0.14$). Thus, plasma testosterone levels do not account for any additional variance not already accounted for by collecting seasons.

We further examined the relationship between plasma testosterone levels and time in captivity. There was a significant decrease in levels with increased time in captivity in reproductive males (ANOVA $p = 0.02$, $R^2 = 0.19$, Figure 2.3B), but no significant change in levels over time in captivity in non-reproductive males pooled across 2009 and 2010 (ANOVA $p = 0.57$, $R^2 = 0.04$, Figure 2.3C). Based on the male reproductive-related decrease in testosterone levels during captivity, the decline in primary afferent tuning in reproductive females after more than two weeks in captivity (Sisneros and Bass, 2003), and evidence for decreased reproductive vocal excitability after more than two weeks in captivity (A. Bass and T. Rubow, unpublished observations), we divided the 2009 reproductive male population into two groups,

Figure 2.3
Plasma testosterone levels vary with saccular physiology. (A) Log transformed plasma testosterone (T) levels in male midshipman from reproductive/ summer season of 2009 and non-reproductive/ winter seasons of 2009 and 2010. Data plotted as means ± SE. * denotes a statistically significant ($p < 0.05$) difference between groups. Log transformed plasma testosterone (T) levels in male midshipman as a function of time spent in captivity in (B) summer 2009 and (C) winter 2009 and 2010 animals.

Figure 2.3 (continued)



those kept in captivity less than (mean 7 ± 2 days) or greater than (mean 21.6 ± 5.0 days) 15 days. Evoked saccular potentials were recorded from 16 reproductive type I males, 12.2 - 17.6 cm SL (mean SL = 14.4 ± 1.6 cm), that had been in captivity greater than 15 days. There was no significant difference in either thresholds (multi-level repeated-measures model: between-subject factor captive duration $p = 0.49$, Figure 2.4A) or iso-level responses of evoked saccular potentials (multi-level repeated-measures model: between-subject factor captive duration $p = 0.41$, Figure 2.4B) between reproductive males kept in captivity less than or greater than 15 days.

Plasma testosterone levels in non-reproductive/winter and reproductive/summer males were further analyzed to see if they correlated with either standard length (SL) or GSI (see materials and methods for calculation). GSI levels peak during the pre-reproductive period and decline during the reproductive season (Sisneros et al., 2008). For non-reproductive animals, GSI values were log transformed for statistical analysis because of unequal variances due in part to a relatively small sample size. In males collected during the non-reproductive /winter seasons of 2009 and 2010 there was a significant positive correlation between GSI and plasma testosterone (ANOVA, $p = 0.03$, $R^2 = 0.44$, Figure 2.5A), but no significant correlation between either SL and plasma testosterone (ANOVA, $p = 0.60$, $R^2 = 0.03$, Figure 2.5B) or SL and GSI (ANOVA, $p = 0.32$, $R^2 = 0.11$, Figure 2.5C). Like winter males, reproductive males showed a significant positive correlation between GSI and plasma testosterone (ANOVA, $p = 0.006$, $R^2 = 0.08$, Figure 2.5D) that was independent of time in captivity ($p = 0.53$). Further, there was no significant relationship between SL and plasma testosterone in reproductive males (ANOVA, p =

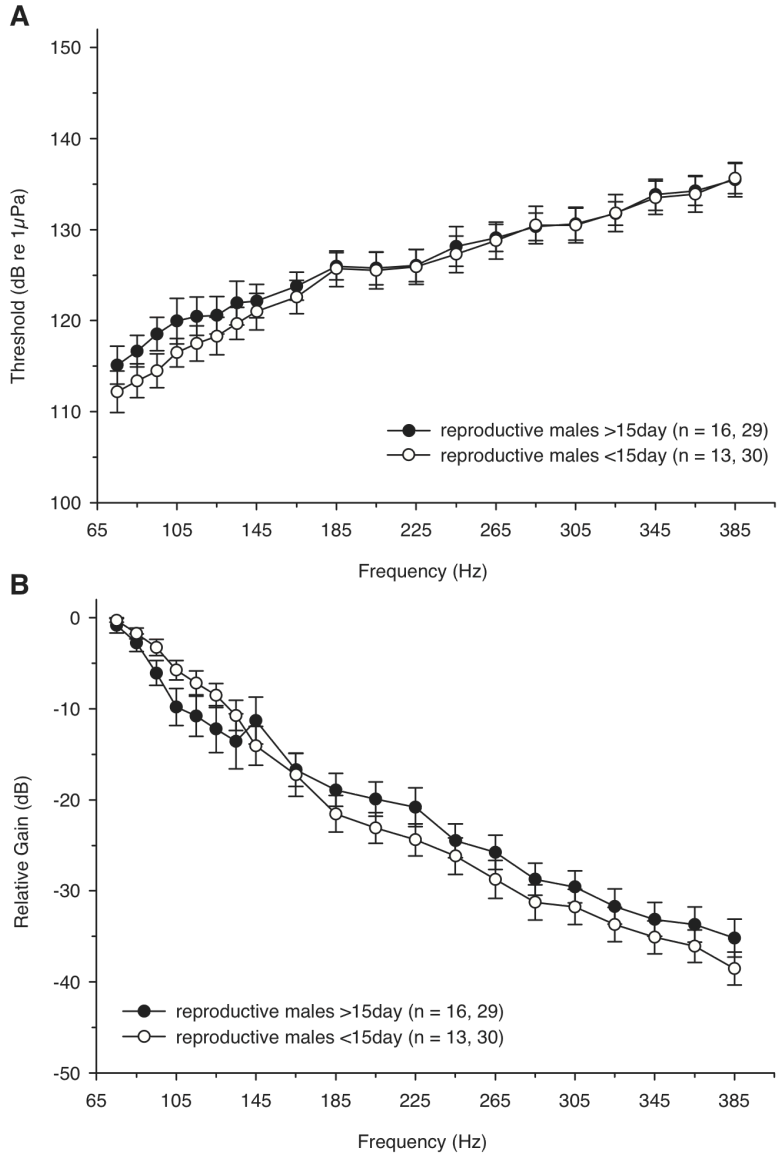


Figure 2.4
(A) Auditory threshold tuning curves for reproductive male midshipman kept in captivity for > or < 15 days based on evoked saccular potentials. (B) Evoked potentials recorded from the saccule of reproductive male midshipman kept in captivity for > or < 15 days in response to iso-level tones of 130 dB (re 1 μPa). Data were normalized with a relative gain value of 0 dB assigned to the peak response for each recording with all other responses expressed as relative dB re the frequency with the largest response. For both (A) and (B) data are plotted as means ± 95 % confidence limit (CL) and the number of animals and records are indicated in parentheses.

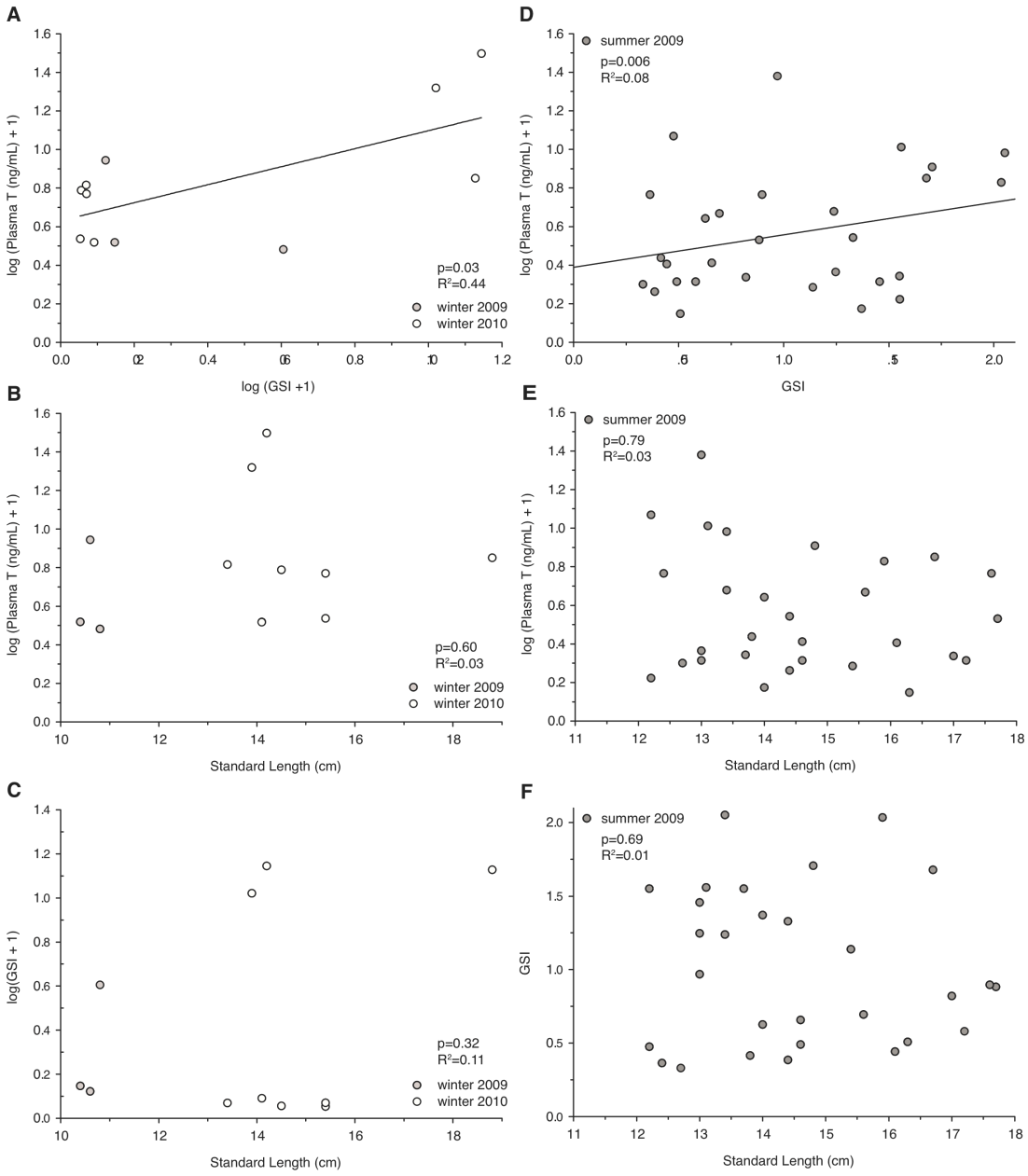


Figure 2.5

Log transformed plasma testosterone (T) levels in non-reproductive/ winter male midshipman versus (A) log transformed gonadosomatic index (GSI) and (B) standard length. (C) Log transformed GSI of winter male midshipman versus standard length. Log transformed plasma T levels in reproductive/ summer male midshipman versus (D) GSI and (E) standard length. (E) GSI of reproductive/ summer male midshipman versus standard length.

0.79, $R^2 = 0.03$, Figure 2.5E), but a significant interaction between SL and time in captivity ($p = 0.004$). This interaction resulted in a negative correlation between standard length and plasma testosterone levels in animals in captivity for less than 15 days. Animals in captivity greater than 15 days showed a positive correlation between standard length and plasma testosterone levels. There was no significant relationship between SL and GSI in reproductive males (ANOVA, $p = 0.69$, $R^2 = 0.01$, Figure 2.5F). Despite the positive correlation between GSI and plasma testosterone, there was no significant difference in GSI between the three collecting seasons (ANOVA, $p = 0.08$). Like plasma testosterone levels, GSI had no significant effect on thresholds ($p = 0.65$) indicating GSI did not account for any variance not already accounted for by collecting season.

Discussion

The primary goal of this study was to test the hypothesis that male midshipman fish, like females, undergo seasonal changes in frequency sensitivity of auditory saccular hair cells concurrent with seasonal changes in gonadal state and circulating levels of steroid hormones. We report two significant new findings. First, type I males, who build nests in the intertidal zone from where they acoustically court females, undergo seasonal changes in saccular physiology statistically indistinguishable from that of females. This is consistent with evidence from underwater playbacks showing both type I males and females using the type I male advertisement call (hum) to localize nests (McKibben and Bass, 1998). Thus, seasonal auditory plasticity is apparently a species rather than a sex-typical trait; we expect type II sneak/satellite spawning males

to exhibit the same phenotype as they too show positive phonotaxis to acoustic playbacks of advertisement calls (McKibben and Bass, 1998). Second, we unexpectedly collected a population of type I males from offshore habitats during non-nesting months in transition from a non-reproductive to reproductive state, characterized by elevated plasma testosterone levels and testes with both mature and immature sperm (Sisneros et al., 2004b). These males had saccular hair cell thresholds intermediate between non-reproductive and reproductive males. This provides essential evidence tying naturally occurring seasonal plasticity in saccular physiology to changing reproductive status, including gonadal recrudescence and circulating steroid levels.

Iso-Level Responses

As with thresholds, iso-level responses of evoked saccular potentials in female midshipman showed reproductive state-dependent variation. The consistency of the results with those previously reported (Sisneros, 2009) demonstrates the robustness of seasonal auditory plasticity as well as the reliability of the recording technique by multiple users in different laboratory settings using similar hardware configurations. While seasonal plasticity in thresholds is consistent between males and females, the relationship is lacking for iso-level response data. We propose that iso-level responses are not valid for seasonal comparisons. Iso-level responses were recorded in this and the earlier study of females (Sisneros, 2009) only at 130 dB re 1uPa. For non-reproductive animals, frequencies above 145 Hz had thresholds above 130 db, while reproductive frequencies greater than 265-285 Hz fell below the same cut-off (vertical

arrows, Figure 2.2A, C). Thus, iso-level responses recorded at 130 dB in non-reproductive and reproductive animals are sub-threshold above 145 and 265-285 Hz, respectively. It is at frequencies greater than 145 Hz that iso-level responses at 130 dB begin to diverge between non-reproductive and reproductive females. When using iso-level responses of evoked saccular potentials to quantitatively distinguish auditory function between seasons, one should either record responses at a stimulus amplitude sufficient to elicit supra-threshold responses at all frequencies or limit the analysis only to frequencies with supra-threshold responses when only using 130 dB stimuli, We suggest that threshold data is the more robust quantitative measure of saccular hair cell responses as it inherently limits itself to analysis of supra-threshold responses and is internally referenced to the background noise of a given animal and recording site, reducing variability between individuals and/or recordings.

The above conclusion regarding the validity of 130 dB iso-level responses moves towards resolving a paradox previously raised for females, namely frequency-dependent seasonal plasticity of iso-level responses, but frequency-independent plasticity in saccular thresholds (Sisneros, 2009). The current study helps resolve this quandary in two ways. First, we propose that the frequency-dependence of iso-level response plasticity is an artifact of the recording technique as described above. Further, while a prior study (Sisneros, 2009) reported no significant interaction between frequency and reproductive state when examining thresholds, the present study showed a significant interaction for both females and type I males (Figure 2.2E). This interaction was more apparent in male threshold data (Figure 2.2C) at frequencies >145 Hz where seasonal differences are greater. Taken together, these lines of

evidence suggest that regardless of the validity of 130 dB iso-level response data, there is a frequency dependence of seasonal plasticity in saccular thresholds.

Resolving this previous conflict in saccular physiology data shows that, like primary auditory afferents (Sisneros and Bass, 2003; Sisneros et al., 2004a), saccular hair cells show frequency dependence in their seasonal plasticity with greater differences occurring at frequencies overlapping the upper harmonics of male calls. This is consistent with the proposed behavioral role of seasonal plasticity in peripheral auditory function enabling detection of higher harmonics (see Figure 2.1A, B) in shallow water (also see Fine and Lenhardt, 1983; Bass and Clark, 2003; Sisneros and Bass, 2003). Similar to hair cell recordings (Figure 2.2A, C), auditory afferents in non-reproductive females had weak responses at 130 dB re 1μPa at frequencies above 140 Hz with the greatest enhancement among reproductive females at frequencies > 140 Hz and up to ~ 340 Hz (Sisneros and Bass, 2003). Hence, the frequency range over which auditory afferent plasticity is greatest, namely > 145 Hz, parallels that for saccular thresholds (Figure 2.2E, F). We propose that seasonal changes in afferent physiology, specifically the frequency dependence of differences > 145 Hz, largely if not entirely reflect plasticity in auditory hair cell function.

Daily Rhythms of Vocal-Acoustic Communication

Unlike the vocal motor system (Rubow and Bass, 2009), we found no photoperiod-dependent sensitivity in any measures of evoked saccular potentials in reproductive type I males. The results may reflect the divergent demands on vocal and auditory systems. While reproductive type I males only produce long-duration (min to > 1h)

vocalizations at night (Brantley and Bass, 1994; Bass et al., 1999), they produce brief grunts (see Figure 2.1B) at all times of the day (Cohen and Winn, 1967; Brantley and Bass, 1994). Thus, although midshipman tend to be more quiescent in general during the light part of the photoperiod (photophase; Bass, 1996), the auditory system is likely subjected to a similar set of constant demands, resulting in a lack of daily rhythm in its sensitivity. Although our experiment argues against the presence of daily rhythms in intrinsic saccular physiology, we cannot rule out the alternative hypothesis that photoperiod-related changes in peripheral physiology depend on acute shifts in central efferent activity that would have been masked by the paralytic, pancuronium bromide, used in our preparation that acts by blocking nicotinic acetylcholine receptors also present at the efferent-hair cell synapse (Glowatzki and Fuchs, 2000). Efferent action on hair cell and afferent physiology in fish lasts on the order of milliseconds (Furukawa, 1981) to more than one second (Boyle et al., 2009) in the saccule and semicircular canals, respectively. Thus, any daily rhythm in saccular efferent activity would have to occur over the time course of pancuronium bromide blockade of efferent-hair cell synapses during the *in vivo* preparation used here.

Timing of Annual Reproductive Rhythms

The observation that type I males collected by trawl in December 2009 were further along in the transition from non-reproductive to reproductive states as compared to males collected in February 2009 demonstrates the year-to-year variability in the midshipman reproductive cycle. Previous studies on circulating steroid levels as well as gonadal condition conducted over a two year period (2001-2002) identified

December through February as a non-reproductive period and March through April as the pre-nesting transitional state (Sisneros et al., 2004b). Plasma steroid levels, testis morphology, and auditory physiology all indicated that animals were already in a pre-nesting condition by December 2009. The variability in the annual onset of transition to a reproductive state and subsequent changes to the vocal-acoustic system, including the auditory inner ear and vocal motor system, suggests an equally variable external circannual trigger. The occurrence of El Niño conditions during the non-reproductive / winter season of 2010 (Lee and McPhaden, 2010) suggests warming ocean water temperatures may affect the midshipman breeding population as has been demonstrated in other fish stocks (Stenseth et al., 2002). Animals may begin the transition from non-reproductive to reproductive state and invest in gonadal development based on internal somatic cues such as nutritional state (Carrillo et al., 2009), which could vary year-to-year based on food availability due to environmental changes such as water temperature. Regardless of what cues the animal to transition from non-reproductive to reproductive states, this study points out the importance of using a variety of metrics including gonad morphology and circulating steroid levels in determining the reproductive state of the animal and that collection date alone is not a foolproof indicator of reproductive state for this species.

Seasonal Plasticity in Male Saccular Physiology, Gonadal Condition and Plasma Testosterone Levels

To our knowledge, this is the first study to demonstrate seasonal plasticity in auditory hair cell physiology in male fish. A prior study of the Hawaiian sergeant damselfish

(*Abudefduf abdominalis*) reported no seasonal differences in auditory evoked potentials (AEPs) in either sex (Maruska et al., 2007). Similarly, the Lusitanian toadfish (*Halobatrachus didactylus*), a member of the same family as midshipman (*Batrachoididae*), does not exhibit seasonal plasticity in saccular thresholds (Vasconcelos et al., 2010). This dichotomy may reflect differences between species that remain on or near their shallow water breeding grounds (Lusitanian toadfish: Amorim et al., 2006; damselfish: Maruska et al., 2007) and those that migrate from shallow breeding grounds to deeper offshore waters (midshipman, Sisneros et al., 2004b) where the acoustic cutoff frequency is lower (Bass and Clark, 2003).

While previous studies have shown reproductive state-related changes in saccular physiology, it was assumed this plasticity was influenced by steroids (Sisneros, 2009) as is the case with saccular afferents (Sisneros et al., 2004a). The current study is the first to provide evidence that the seasonal state of saccular hair cells correlates with seasonal changes in circulating steroid (testosterone) levels. While onset of reproductive auditory physiology correlates with increasing plasma testosterone levels, elevated testosterone levels do not appear to be necessary to maintain this phenotype for up to a month (Figures 2.3B, 2.4). However, circulating plasma testosterone levels may not reflect local levels within the peripheral auditory system. It remains to be determined whether steroids, including testosterone and its metabolites (estradiol, 11-ketotestosterone; see Bentley, 1998) are necessary and sufficient to induce seasonal changes in saccular hair cell function. The presence of both androgen (Forlano et al., 2010) and estrogen (Forlano et al., 2005) receptors in the saccular epithelium of midshipman as well as the enzyme aromatase, which

converts testosterone to estradiol, in the saccular branch of the eighth nerve (Forlano et al., 2001) suggests testosterone may act both directly via androgen receptors and indirectly via estrogen receptors on saccular hair cells as proposed for saccular afferents (Sisneros et al., 2004a). This pathway would work similarly in both males and females despite sex differences in circulating steroids (estradiol in females, 11-ketotestosterone in type I males)(Brantley et al., 1993a; Knapp et al., 1999; Sisneros et al., 2004b).

Mechanisms for Saccular Hair Cell Plasticity

While several studies describe seasonal changes in midshipman auditory physiology (Sisneros and Bass, 2003, current study; Sisneros, 2009), little progress has been made in identifying the mechanism(s) of such plasticity save for identifying that it can be induced, at least at the afferent level, by 3 - 5 week steroid implants (Sisneros et al., 2004a). The occurrence of estrogen receptors in the auditory epithelium of vertebrates including fishes (midshipman, (Forlano et al., 2005); cichlid, (Maruska and Fernald, 2010)), songbird (Noirot et al., 2009), rodents (Stenberg et al., 1999) and humans (Stenberg et al., 2001)) suggests a widespread occurrence of steroid-dependent auditory plasticity among vertebrates. Several mechanisms might account for this plasticity (also see Sisneros et al., 2004a). The first is hair cell addition. Corwin (1983) correlated auditory hair cell addition into adulthood in sharks with increased auditory sensitivity. Pre-nesting increases in local estrogen due to either circulating estrogen (females) or local aromatization of circulating testosterone (both sexes) within the saccule may trigger hair cell proliferation prior to the reproductive season as has been

shown in midshipman vocal muscle (Brantley et al., 1993b). While hair cell addition to the saccule of reproductive midshipman might account for a general decrease in thresholds compared to non-reproductive animals, the properties of these new hair cells would have to be such that a higher proportion of them have higher best frequencies in order to account for the frequency dependence of seasonal changes in saccular thresholds.

Seasonal changes in the ionic composition of saccular endolymph could result in changes to the ionic driving forces for currents that play roles in electrical tuning of hair cells. It has been proposed in mammals that estrogen receptors alpha and beta, both of which are present in cells of the stria vascularis and Reissner's membrane involved in ion and fluid homeostatis, may alter ionic gradients within the inner ear (Stenberg et al., 1999). Estrogen receptor mRNA is expressed in supporting cells with similar functions within the saccular epithelium of midshipman (Forlano et al., 2005, see Forlano et al., 2010 for androgen receptor). Estrogen inhibits ion transport through I_{Ks} channels in the stria vascularis of gerbils via non-genomic mechanisms which potentially include membrane estrogen receptors or direct interactions with ion channel protein complexes (Lee and Marcus, 2001). While the time course of seasonal changes in midshipman suggests a long-term, transcriptionally-dependent response, estrogen may be changing ion channel expression to produce protracted changes in endolymph composition.

A potential mechanism with the appropriate time scale and frequency specificity is the seasonal change in expression of ion channels regulating the electrical resonance and thus frequency selectivity of auditory hair cells. As noted

previously (Sisneros and Bass, 2003), a similar mechanism for steroid-dependent frequency sensitivity has been proposed for electroreceptors in weakly electric fish (Zakon and Meyer, 1983). One of the primary determinants of electrical resonant frequencies of vertebrate auditory hair cells is the expression of large conductance, calcium-activated potassium (BK) channels (Fettiplace and Fuchs, 1999). The frequency range at which BK channel diversity accounts for electrical tuning in turtles and other non-mammalian vertebrates (Fettiplace and Fuchs, 1999) encompasses the frequency range of midshipman hearing. BK channels play an analogous role in the membrane oscillations of saccular hair cells in goldfish (Sugihara and Furukawa, 1989; Sugihara, 1994), and have been identified as a major outward current in saccular hair cells of toadfish (*Opsanus tau*, Steinacker and Romero, 1991), a species in the same family (Batrachoididae) as midshipman (Nelson, 2006). The rapid kinetics of both activation and deactivation of BK currents are necessary for electrical resonance in toadfish saccular hair cells (Steinacker and Romero, 1992).

The *slo1* gene that encodes the pore-forming α-subunit of BK channels is duplicated in midshipman and both genes are expressed in the saccular epithelium (Rohmann et al., 2009). Expression of transcripts of one of these two genes (*slo1a*) is upregulated in saccular epithelium of reproductive compared to non-reproductive adults (Rohmann and Bass, 2010). Whether upregulation of *slo1* expression during the reproductive season results in changes in BK currents, and is regulated by testosterone (e.g., Mahmoud and McCobb, 2004) and / or estrogen (e.g., Holdiman et al., 2002; Zhu et al., 2005) to account for steroid-dependent physiological plasticity are questions we are currently investigating.

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Appendix

While the studies outlined in Chapter 2 did not involve any direct experimental manipulation of steroid levels in order to establish a causal relationship between fluctuating steroid levels and saccular hair cell physiology, we conducted two separate studies in an attempt to determine whether gonadally derived steroids are necessary for maintaining a reproductive auditory phenotype (Experiment 1) and sufficient to induce a transition from a non-reproductive to reproductive phenotype (Experiment 2).

Methods

Experiment 1

Adult female midshipman (n=11) were collected from nests in California in July 2009 during the same period as reproductive females used in the studies described above. Within a week of arriving at Cornell University, animals were gonadectomized and received an intraperitoneal implant. Implants consisted of 1cm length of plastic tubing (2mm outer diameter, 1.25mm inner diameter) filled with either coconut oil (Sigma, St. Louis, MO) or coconut oil containing 0.5mg estradiol (Sigma, St. Louis, MO) / ml oil. Oil remained solid at room temperatures and below at which midshipman are housed and experiments are conducted. Following surgery animals were housed as normal until saccular physiology was performed 33-39 days later as outlined in methods in the preceding chapter.

Experiment 2

Adult midshipman (both females (n=5) and type I males (n=7)) were taken from the same February 2009 trawl as non-reproductive animals used in the experiments described in the preceding chapter. Approximately 4-6 weeks following arrival at Cornell University, animals were received either coconut oil (control) or 0.5mg testosterone (Sigma, St. Louis, MO)/ ml oil intraperitoneal implants made as described above. Following surgery animals were housed as normal until saccular physiology was performed 26-30 days later as outlined in methods in the preceding chapter. Following auditory physiology, blood was collected from the heart and testosterone assays were performed on plasma as described in the preceding chapter.

Results

Experiment 1

There was no overall effect of treatment on saccular thresholds (multi-level repeated-measures model: between-subject factor treatment, $p = 0.78$, Figure 2.6). Niether gonadectomized (GDX) plus control implant (Tukey-Kramer HSD, $p = 0.78$) nor GDX plus estradiol implant (Tukey-Kramer HSD, $p = 0.99$) females had significantly different thresholds from intact females (data on intact females from above chapter).

Experiment 2

There was an overall significant effect of treatment on thresholds (multi-level repeated-measures model: between-subject factor treatment, $p = 0.02$, Figure 2.7a) with testosterone treated animals having significantly lower thresholds from non-reproductive animals that received no implant (Tukey-Kramer HSD, $p = 0.04$). Note that data on non-reproductive animals receiving no implant is pooled from 2009 non-reproductive type I males and females from the preceding chapter. Animals receiving control implants had thresholds that did not differ from animals that received either no implant (Tukey-Kramer HSD, $p = 0.06$) or testosterone implant (Tukey-Kramer HSD, $p = 0.97$). Radioimmunoassays for testosterone confirmed that testosterone implants significantly increased plasma testosterone levels compared to both control (Tukey-Kramer HSD, $p = 0.0008$) and no implant (Tukey-Kramer HSD, $p = 0.0001$) groups (Figure 2.7b). While samples sizes were too small for robust statistical analyses (control: 3 females, 3 type I males; testosterone: 2 females, 4 type I males) there was no marked different in thresholds between sexes for in animals receiving either control

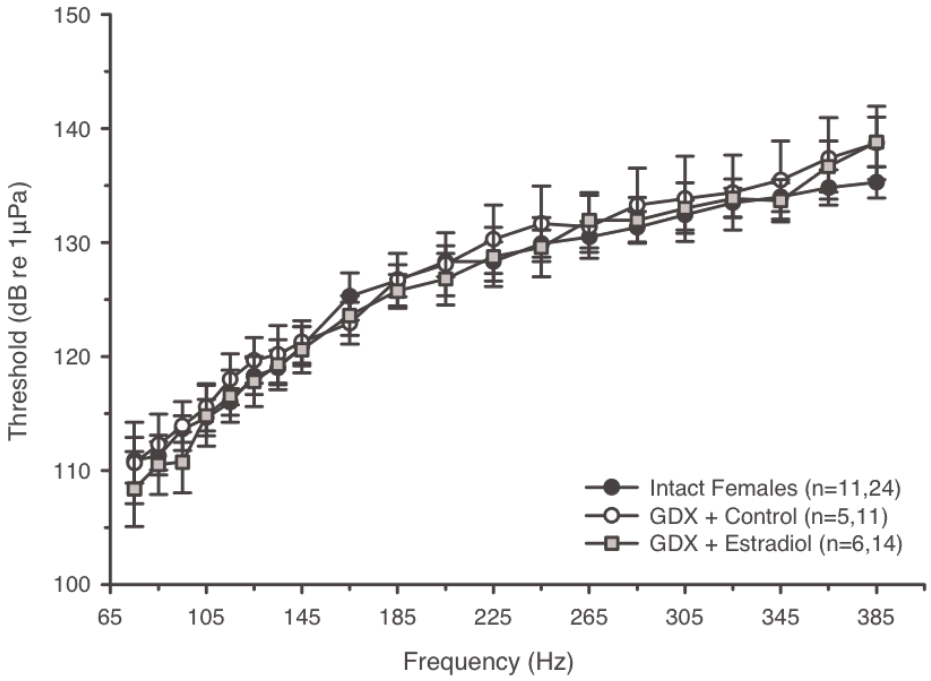

Figure 2.6
Gonadally secreted steroids are not necessary to maintain a reproductive saccular physiology phenotype. There was no overall effect of treatment on thresholds (multi-level repeated-measures model: between-subject factor treatment, $p = 0.78$). Neither gonadectomized (GDX) plus control implant (Tukey-Kramer HSD, $p = 0.78$) nor GDX plus estradiol implant (Tukey-Kramer HSD, $p = 0.99$) females had significantly different thresholds from intact females. Error bars indicated 95% confidence intervals. Samples sizes are reported as number of animals, number of total recordings.

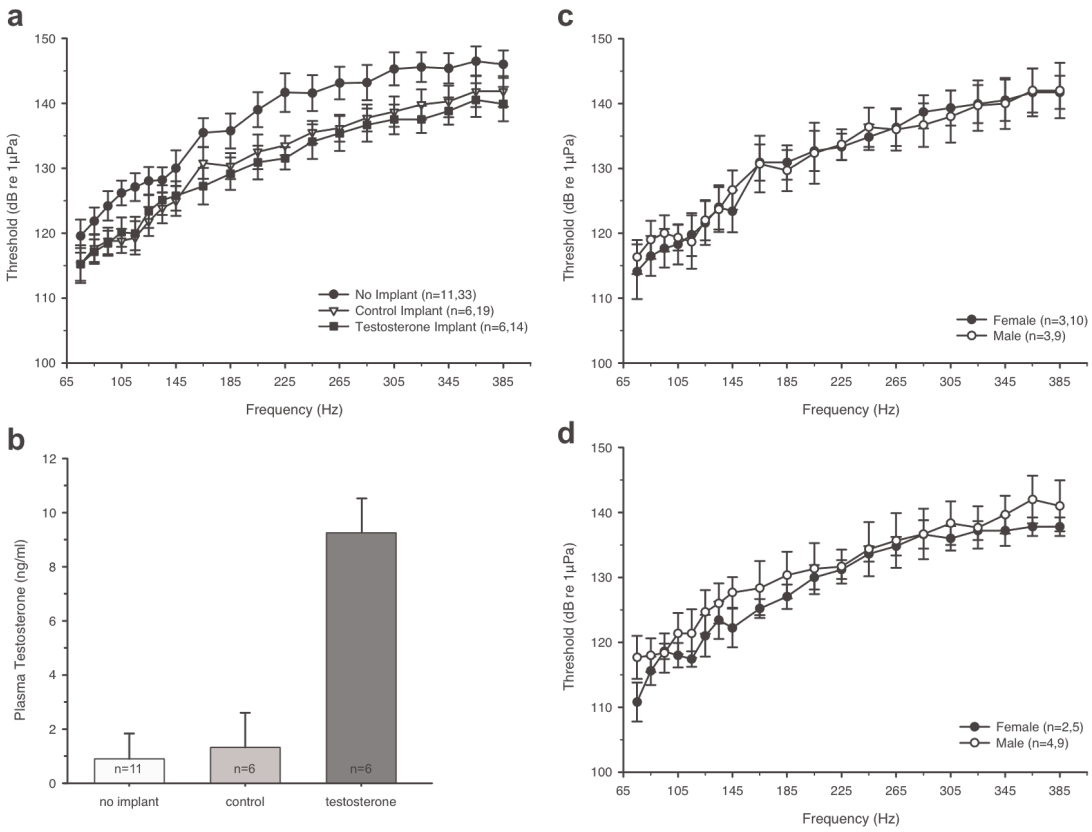

Figure 2.7

Testosterone implants significantly decreased saccular thresholds in intact non-reproductive midshipman. (**a**) There is an overall significant effect of treatment on thresholds (multi-level repeated-measures model: between-subject factor treatment, $p = 0.02$) with testosterone treated animals having significantly lower thresholds from non-reproductive animals that received no implant (Tukey-Kramer HSD, $p = 0.04$). Animals receiving control implants had thresholds that did not differ from animals that received either no implant (Tukey-Kramer HSD, $p = 0.06$) or testosterone implant (Tukey-Kramer HSD, $p = 0.97$). (**b**) Testosterone implants significantly increased plasma testerone levels over both control (Tukey-Kramer HSD, $p = 0.0008$) and no implant (Tukey-Kramer HSD, $p = 0.0001$) groups. Error bars indicate standard errors. There was notable difference in thresholds between sexes in either (**c**) control or (**d**) testosterone treated individuals. For (**a,c,d**) error bars indicated 95% confidence intervals and samples sizes are reported as number of animals, number of total recordings.

(Figure 2.7c) or testosterone (Figure 2.7d) implants.

Discussion

Experiment 1

We report that gonadally synthesized steroids are not necessary for maintaining a reproductive saccular hair cell physiology phenotype in female midshipman. If gonadal steroids, particularly estradiol which has been shown to be sufficient for inducing a reproductive phenotype in eighth nerve afferents in non-reproductive females (Sisneros et al 2004a), were both necessary and sufficient to maintain a reproductive phenotype we would have predicted GDX plus estradiol animals would have retained a reproductive phenotype while GDX plus control animals would begin to transition toward a non-reproductive phenotype. Given that we have found no sex differences in seasonal plasticity of saccular hair cells, we would predict that a similar experiment conducted on type I males would produce similar results.

While gonadal steroids may not be necessary for maintaining reproductive saccular hair cell physiology, steroid synthesis in other parts of the body including the brain and eighth nerve (Forlano et al 2005), may provide sufficient levels of steroids to maintain a reproductive phenotype. Use of steroid receptor antagonists and/or drugs to block the action of aromatase and other steroidogenic enzymes may serve to further address this hypothesis. Alternatively, the time period over which we conducted our experiment may not be sufficient to induce a transition to a non-reproductive phenotype. Our approximately month long time period from gonadectomy to physiology was based on the observation that one month treatment of non-

reproductive females with steroid implants could induce a reproductive phenotype in eighth nerve afferents (Sisneros et al 2004a). The reverse transition may simply take longer to occur or hair cell plasticity occurs over a time course with different duration than afferent plasticity.

Experiment 2

While our data indicate a statistically significant effect of testosterone in lowering saccular thresholds in non-reproductive animals, shifting towards a reproductive saccular hair cell physiology phenotype, the nearly significant ($p=0.06$) effect of control implant animals compared to no implant questions this conclusion. If conducted on a larger sample size our data would likely reveal that both testosterone and control implants resulted in a significant decrease in thresholds. One explanation for the apparent transition in saccular physiology from a non-reproductive towards a reproductive-like state is continued gonadal recrudescence in both implant groups. We did not gonadectomize these animals at the time of implantation to reduce the risk of post-operative complication in winter animals, which tend to be less robust than summer animals due in part to in the increased stress of collection by trawling compared to hand collection from nests. The intact gonads in these animals may have begun to secrete higher levels of steroids as was observed in the winter 2010 males described in the preceding chapter which had both transitional gonads and saccular physiology consistent with a transition from a non-reproductive towards a reproductive state. Our physiology data on both testosterone and control implant animals in Figure A.2a is reminiscent of this group. It should be noted that unlike

winter 2010 males, control implant animals did not show a significant increase in plasma testosterone levels. It remains possible that other gonadal steroids may have been produced at higher levels in control and testosterone implanted animals. A transition toward reproductive state is even more likely in these implanted animals as physiology on these animals was conducted in mid-April when animals have been collected in reproductive condition from nest sites in both California and Washington. To truly test whether steroids such as testosterone and estradiol are sufficient to induce seasonal plasticity at the hair cell level as has been shown for eighth nerve afferents (Sisneros et al 2004a) animals in a truly non-reproductive state should be selected and gonadectomized to remove this potential source of steroids.

CHAPTER 3

CALCIUM-ACTIVATED POTASSIUM (BK) CHANNELS ARE ENCODED BY DUPLICATE *slo1* GENES IN TELEOST FISHES

Abstract

Calcium-activated, large conductance potassium (BK) channels in tetrapods are encoded by a single *slo1* gene, which undergoes extensive alternative splicing. Alternative splicing generates a high level of functional diversity in BK channels that contributes to the wide range of frequencies electrically tuned by the inner ear hair cells of many tetrapods. To date, the role of BK channels in hearing among teleost fishes has not been investigated at the molecular level, although teleosts account for approximately half of all extant vertebrate species. We identified *slo1* genes in teleost and non-teleost fishes using PCR and genetic sequence databases. In contrast to tetrapods, all teleosts examined were found to express duplicate *slo1* genes in the central nervous system, while non-teleosts that diverged prior to the teleost whole genome duplication event express a single *slo1* gene. Phylogenetic analyses further revealed that while other *slo1* duplicates were the result of a single duplication event, an independent duplication occurred in a basal teleost (*Anguilla rostrata*) following the *slo1* duplication in teleosts. A third, independent *slo1* duplication (autotetraploidization) occurred in salmonids. Comparison of teleost *slo1* genomic sequences to their tetrapod orthologue revealed a reduced number of alternative splice sites in both *slo1* co-orthologues. For the teleost *Porichthys notatus*, a focal study species that vocalizes with maximal spectral energy in the range electrically tuned by

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BK channels in the inner ear, peripheral tissues show the expression of either one (e.g. vocal muscle) or both (e.g. inner ear) *slo1* paralogues with important implications for both auditory and vocal physiology. Additional loss of expression of one *slo1* paralogue in non-neural tissues in *P. notatus* suggests that *slo1* duplicates were retained via subfunctionalization. Together, the results predict that teleost fish achieve a diversity of BK channel subfunction via gene duplication, rather than increased alternative splicing as witnessed for the tetrapod and invertebrate orthologue.

Introduction

Calcium-activated, large conductance potassium (BK) channels are responsible for the currents that contribute to the electrical resonance defining the frequency tuning of hair cells in non-mammalian vertebrates including fish, amphibians, reptiles, and birds (Fettiplace and Fuchs, 1999). The α-subunit of BK channels is encoded by a single *Slo1* (*KCNMA1*) gene in tetrapods (Salkoff et al., 2006). Alternative splicing of *Slo1*, along with post-translational modification and association with β-subunits, accounts for variation in BK channel properties that shape variable electrical resonance between hair cells in nonmammals (Jones et al., 1999; Ramanathan et al., 1999). While frequency tuning of the peripheral auditory system in mammals is achieved through mechanical as opposed to electrical mechanisms, BK channels are necessary for normal function of the cochlea, the primary end organ of hearing in mammals. Knockouts of *Slo1* in mice result in progressive hearing loss (Ruttiger et al., 2004) and impaired temporal precision of electrical signals in auditory afferents (Oliver et al., 2006).

BK channel currents have been identified as one of the major outward currents in the hair cells of teleost fish (Sugihara and Furukawa, 1989; Steinacker and Romero, 1991, 1992). Beisel *et al*. (2007) reported the genomic organization of a *slo1* gene in the zebrafish *Danio rerio* from sequences deposited in electronic databases, while Lionetto *et al*. (2008) examined the expression of BK channel transcripts in the intestinal epithelium of the European eel *Anguilla anguilla*. The current study, however, is the first to examine the expression of genes encoding BK channels in the nervous system of this group of vertebrates which accounts for nearly half of all living vertebrate species (Nelson, 2006). We identified two duplicate *slo1* genes encoding BK channels in several teleosts and then conducted phylogenetic analyses to determine the timing of the duplication(s) of *slo1* genes in teleosts. Next, we examined the genomic sequences of teleost *slo1* genes to determine whether each *slo1* co-orthologue has the same complement of splice sites as the orthologous gene in tetrapods, and calculated the relative rates of evolution between *slo1* paralogues. Finally, we investigated the tissue-specific expression patterns of *slo1a* and *slo1b* in the plainfin midshipman fish (*Porichthys notatus*) that produce vocalizations with spectral content mainly below 1 kHz. Our initial goal was to identify the gene(s) encoding BK channels in the peripheral auditory system of midshipman because the saccule, the main auditory division of the inner ear, and the eighth nerve that innervates the saccule (McKibben and Bass, 1999; Sisneros and Bass, 2003; Sisneros et al., 2004; Sisneros, 2007), are most sensitive to 60-400Hz, falling within the range of frequencies electrically tuned for by BK channels (Fettiplace and Fuchs, 1999). However, as reported below, the discovery of two *slo1* genes in midshipman led to the

current study that included distantly related fishes to investigate how widespread this duplication event might be, and when it might have occurred in the evolutionary history of fishes.

Materials and Methods

Taxon Sampling

There are two major lineages of living fishes, the actinopterygians and sarcopterygians (see Nelson 2006). Actinopterygians include the highly speciose teleosts and several smaller groups of non-teleosts, while sarcopterygians include lungfish, coelacanths and tetrapods. We sampled teleosts commonly used in the study of developmental genetics (the zebrafish *Danio rerio* (Nicolson, 2005) and the stickleback *Gasterosteus aculeatus* (Peichel, 2005)), social behavior (the cichlid *Neolamprologus pulcher* (Fitzpatrick et al., 2008)), potassium channels (the rainbow trout *Onchorhynchus mykiss* (Henne and Jeserich, 2004), and auditory neurophysiology (*P. notatus* and the Gulf toadfish *Opsanus beta* (Bass and McKibben, 2003)). The locally available American eel *Anguilla rostrata* was substituted for the European eel *A. anguilla* for which a partial *slo1* sequence has been reported (Lionetto et al., 2008)). A chondrichthyan (the little skate *Leucoraja erinacea*) as well as several non-teleost actinopterygians (the bichir *Polypterus senegalus,* the longnose gar *Lepisosteus osseus*, the bowfin *Amia calva*) were selected to determine whether duplication of the *slo1* gene occurred prior to the teleost whole genome duplication event. The Ensembl genome browser and the National Center for Biotechnology Information (NCBI) databases were used to retrieve *slo1* sequences from tetrapods in which the gene has

been studied extensively (the human *Homo sapiens* ENSG00000156113, the mouse *Mus musculus* ENSMUSG00000063142, the chicken *Gallus gallus* ENSGALG00000004980, the turtle *Trachemys scripta* AF036625) as well as teleosts for which complete or nearly complete *slo1* entries were available (the stickleback *G. aculeatus*, the Japanese rice fish *Oryzias latipes*, the tiger pufferfish *Takifugu rubripes*, the green spotted pufferfish *Tetraodon nigroviridis*, see table 3.1 for Ensembl gene entry numbers). A recently published partial sequence of a *slo1* transcript from the intestinal epithelium of *A. anguilla* was also included in analyses (Lionetto et al., 2008).

slo1 *Cloning and Sequencing*

Whole central nervous system (CNS, spinal cord and/ or brain) RNA was extracted from a single individual for each species except for *N. pulcher* (n=2), *G. aculeatus* (n=3), *P. notatus* (n=3), and *D. rerio* (n=11) for which tissue was pooled from multiple animals, and *T. nigroviridis* and T. *rubripes* for which only Ensembl sequence data was used. The Institutional Animal Care and Use Committee at Cornell University approved all procedures in this study. The samples from *P. notatus* and *O. beta* contained the anterior spinal cord since the vocal-motor circuit extends from the caudal hindbrain into the anterior spinal cord in both species (Bass et al., 1994). Whole CNS RNA was isolated using the Trizol method (Invitrogen), treated with DNase I (Invitrogen) to remove genomic DNA contamination, and reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen) following the manufacturer's protocols. PCR on cDNA from whole CNS was conducted using degenerate primers

Table 3.1
GenBank accession numbers of new *slo1* nucleotide sequences reported in this study.

Class/Infraclass	Order	Family	Taxon	Gene	GenBank Accession No.
Chondrichthyes	Rajiformes	Rajidae	*Leucoraja erinacea*	*slo1*	FJ265726
Actinopterygii	Polypteriformes	Polypteridae	*Polypterus senegalus*	*slo1*	FJ265727
	Lepisosteiformes	Lepisosteidae	*Lepisosteus osseus*	*slo1*	FJ265728
	Amiiformes		*Amia calva*	*slo1*	FJ265723
Teleostei	Anguilliformes	Anguillidae	*Anguilla rostrata*	*slo1a*	FJ265724
				slo1b	FJ265725
Euteleostei	Cypriniformes	Cyprinidae	*Danio rerio*	*slo1a*	FJ269019
				slo1b	FJ269020
	Salmoniformes	Salmonidae	*Oncorhynchus mykiss*	*slo1ai*	FJ269021
				slo1aii	FJ269022
				slo1bi	FJ269023
				slo1bii	FJ269024
	Batrachoidiformes	Batrachoididae	*Porichthys notatus*	*slo1a*	FJ269025
				slo1b	FJ269026
	Batrachoidiformes	Batrachoididae	*Opsanus beta*	*slo1a*	FJ269027
				slo1b	FJ269028
	Gaasterosteiformes	Gasterosteidae	*Gasterosteus aculeatus*	*slo1a*	FJ269029
				slo1b	FJ269030
	Perciformes	Cichlidae	*Neolamprologus pulcher*	*slo1a*	FJ269031
				slo1b	FJ269032

(forward primer: 5'-GGG GAA TTC GGN GAY CCN TGG GAR AAY TTY CAR AAY-3', reverse primer: 5'-GGG AAG CTT NGC RTC RTC NCC YTC YTT CCA RTT CCA-3') designed to the highly conserved pore region based on an alignment of published tetrapod *slo1* sequences corresponding to *H. sapiens* nucleotides 1177-1794 (see figure 3.1). Tetrapod *slo1* sequences were used for primer design because at the time of primer design the teleost *slo1* sequences from Ensembl listed in the previous section were not yet available. PCR amplification was performed with an initial denaturation step of 94°C for 4 minutes followed by 5 cycles of 94°C for 45 sec, 55°C for 45 sec, and 72°C for 2 min, an additional 30 cycles of 94°C for 45 sec, 60°C for 45 sec, and 72°C for 2 min, and a final extension at 72°C for 10 min resulting in a 690 bp product. Because the alternatively spliced exons (10 and 11) spanned by these primers were mutually exclusive in the transcript and were of the same length, this 690 bp product could have contained multiple alternatively spliced transcripts. Primers contained restriction enzyme sites at the 5' end in order to facilitate ligation into Bluescript KS(+) plasmid. The resulting products were subcloned into ultracompetent DH5α cells (*Drosophila* Genomics Resource Center, Indiana University - Nov 18, 2005), plated, grown in liquid culture, miniprepped (Qiagen), and sequenced by the Cornell University Life Sciences Core Laboratory Center using universal primers. Sequences were analyzed and aligned using the Clustal W algorithm in Lasergene (DNASTAR, Inc., Madison, WI, USA).

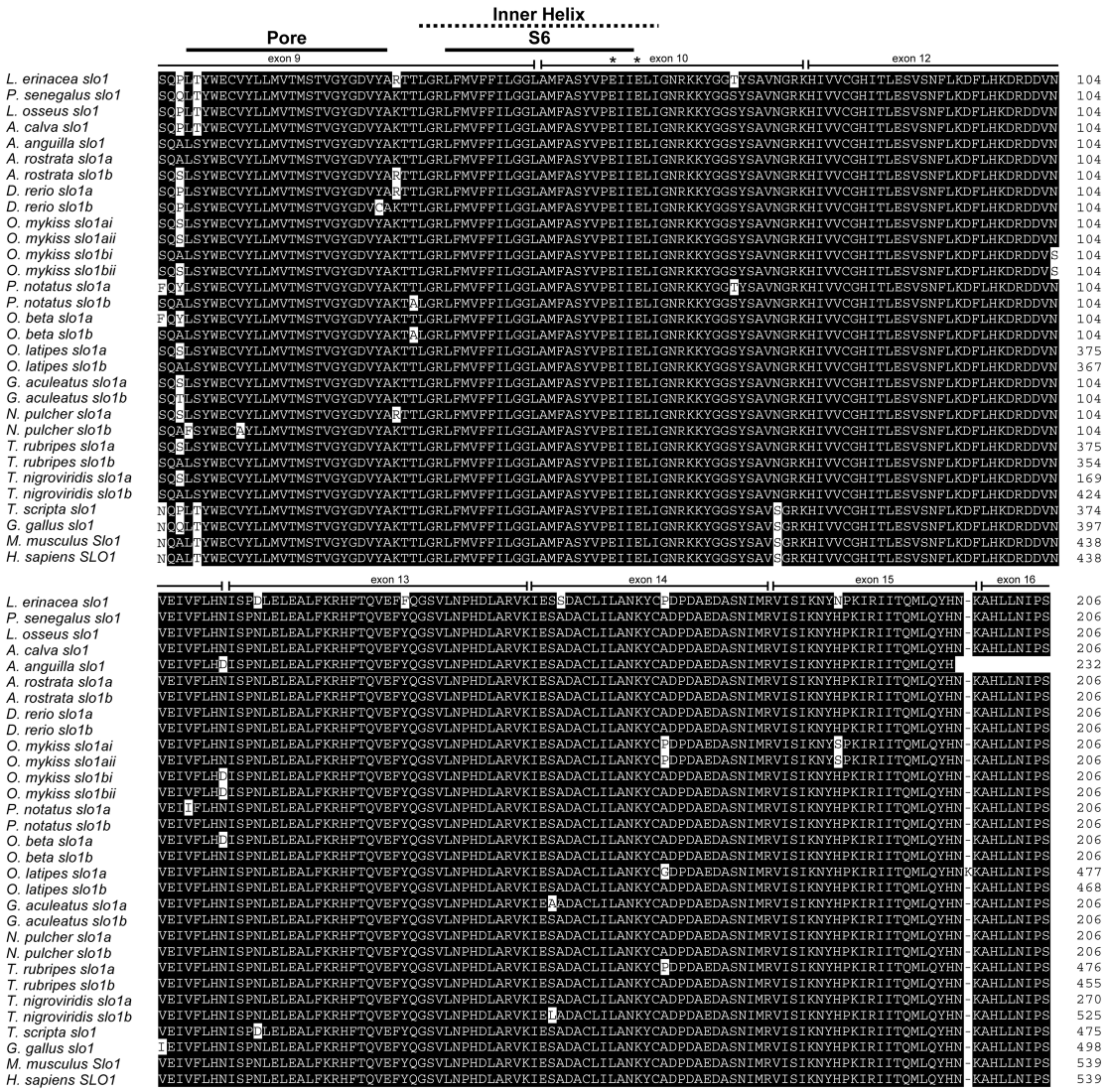


Figure 3.1

Alignment of partial *slo1* protein sequences from species examined. The pore region, sixth membrane-spanning domain (S6), and inner helix of the pore are labeled. Exons beginning with exon 9 are labeled above the corresponding sequence (as numbered by Beisel *et al*. 2007). Note that the sequence skips exon 11 because exons 10 and 11 are mutually exclusive alternatively spliced exons. The glutamic acid residues (E) within the inner helix (*) have been shown to be necessary for the high conductance of BK channels encoded by the slo1 gene. The second of these residues is replaced by an alanine in a commonly occurring splice variant (inclusion of exon 11 in place of 10) expressed in a variety of tissues in the midshipman fish *Porichthys notatus*. Sequences are listed by genus name in phylogenetic order after Nelson 2006.

Genomic Southern Blots

Genomic southern blots were conducted to confirm that the two *slo1* transcripts obtained from *P. notatus* were the products of two genes. The initial *slo1* sequences obtained as described above were expanded upon using an additional degenerate reverse primer (5'-GGG AAG CTT NGG RCA CCA RETG RAA CAT NCC NGT-3') based on the same tetrapod alignment used to design the earlier primer pair. This reverse primer was paired with the forward primer used earlier using the same PCR reaction conditions and the resulting PCR product was subcloned and sequenced as described above to obtain *P. notatus slo1a* and *slo1b* sequences corresponding to *H. sapiens* nucleotides 1177-2475. To prepare specific probes that spanned more divergent sequences than the initial products sequenced that encode the highly conserved pore region of the BK channel, we used the expanded sequence data to design two *P. notatus* species-specific primer pairs corresponding to nucleotides 1886-2248 (exons 16 – 21) in the *H. sapiens* sequence (forward primer 1: 5'-GGG GAA TTC CAT GTT GGC CAA CCT GTT CTC CAT GAG G-3', reverse primer 1: 5'-GGG GGA TCC GGT CAG TGA TGT CAT CAT GAC ACG CTT TAC-3', forward primer 2: 5'-GGG GAA TTC TAT GCT GGC CAA TCT TTT CTC CAT GAG GTC C-3', reverse primer 2: 5'-GGG GGA TCC GTA CAG TGA TGT CAT CAT GAC AAG CTT TAC-3'). Single exons of *slo1* were not sufficiently long to serve as effective templates for random primed probes (templates less than 300 bp work poorly) so we were forced to use templates that spanned multiple exons. PCR was performed on *P. notatus* whole CNS cDNA. PCR amplification was performed with an initial denaturation step of 94°C for 4 minutes followed by 5 cycles of 94°C

for 45 sec, 54.4°C for 45 sec, and 72°C for 1 min, an additional 30 cycles of 94°C for 45 sec, 60°C for 45 sec, and 72°C for 1 min, and a final extension at 72°C for 10 min. The resulting ~350 bp products were subcloned and sequenced as above.

Genomic DNA was extracted from liver of *P. notatus* using the Puregene DNA Purification Kit (Gentra). Ten μg aliquots of DNA were digested with several different restriction enzymes (*BamHI*, *EcoRI*, *HindIII*), run on a 0.8% agarose gel along with the probe templates as a control for probe specificity, and blotted onto a nylon membrane. We selected restriction enzymes that cut relatively infrequently within the target sequence in order to increase the odds that our probes would recognize a single band for each gene. Probes were synthesized from both plasmids using the High Prime DNA labeling Kit (Roche) and [^{32}P] tagged dCTP. The blot was then probed and washed following the methods of Church and Gilbert (1984) and the blot exposed to Kodak BioMax MS film with an intensifying screen.

Qualitative Expression of slo1a *and* slo1b *in* Porichthys notatus *Tissues*

Primers were designed to hybridize to short segments of both *P. notatus slo1a* and *slo1b* with 100% nucleotide identity within the priming site to amplify all *slo1* transcripts in the given cDNA sample, including any splice variants within the region defined by the primers (forward primer: 5'-TTC ATG GTC TTC TTC ATC CT-3', reverse primer: 5'-GAA CAA GGC TTC CAG CTC AAG). These primers amplified a region of *P. notatus slo1a* and *slo1b* corresponding to nucleotides 1279-1545 in the *H. sapiens* sequence. PCR amplification was performed with an initial denaturation step

of 94°C for 4 minutes followed by 35 cycles of 94°C for 45 sec, 46°C for 45 sec, and 72°C for 1.5 min, and a final extension at 72°C for 10 min resulting in 266 bp product.

The 266 bp PCR products were gel extracted and half the volume of each sample (150-350 ng DNA) was digested with 40 units of *XhoI* which digests *slo1*b into two pieces (116 and 150 bp) while leaving *slo1a* intact. Digests were carried out at 37°C for 2 hours. The digested PCR product was run alongside the non-digested volume on a 3% agarose gel in order to determine the relative expression of *slo1a* and *slo1b* within various *P. notatus* tissues including CNS, auditory (saccular) epithelium, vocal muscle, trunk muscle, gill, heart, pituitary, and intestine. In addition, PCR was conducted on sequenced plasmids containing either *slo1a* or *slo1b* to produce known samples of pure *slo1a* and pure *slo1b*. To confirm complete digestion of all *slo1b* without digestion of *slo1a*, 400 ng of pure *slo1a* and pure *slo1b* were digested and run on a gel as described above. Note that more DNA was added to each control digest than for the digests of PCR products (see above) to be certain that the digest conditions were sufficient to cut all *slo1b* in any of the samples digested. The experiment was replicated on cDNA from either a second individual or a different pooled sample in order to confirm the results were not due to individual variability of *slo1* expression. For those tissues that appeared to express exclusively one paralogue (vocal and trunk muscle and gill), the uncut purified PCR product (266 bp band) was sequenced by the Cornell University Life Sciences Core Laboratory Center using the PCR primers in order to confirm the identity of the gel band.

Phylogenetic Analyses

As mentioned above, all nucleotide sequences (618 bp long excluding primer sequences) were aligned using the Clustal W algorithm in Lasergene (DNA*) (figure 3.2). Where available, the full-length nucleotide sequence from the Ensembl database was used in the alignment. In general, there was high sequence identity across the taxa sampled with *slo1* co-orthologues sharing high sequence identity with the single *slo1* gene in chondrichthyan, non-teleost actinopterygians, and tetrapods. Phylogeny was estimated using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) using the facilities of the Computational Biology Service Unit of Cornell University. A general time-reversible model with invariable sites and a gamma distribution for variable rate sites (GTR + I + G) was applied. Four Markov chains of 10,000,000 generations were run sampling every 100th tree. A burn-in of 25% was applied to remove all trees generated in the first quarter of the analysis. From the remaining trees a majority rule consensus tree was generated along with the posterior probabilities. The analysis was replicated to confirm both the consensus tree and the posterior probabilities. Modeltest 3.7 (Posada and Crandall, 1998) was used to confirm that the same model could be applied to both *slo1a* and *slo1b* by analyzing *slo1* + *slo1a* and *slo1* + *slo1b* as two separate data sets.

The resulting consensus tree was edited in Mesquite 2.5 (Maddison and Maddison, 2008) to reflect tree topologies of alternative hypotheses explaining the duplication of *slo1*. These hypothetical trees were compared to the consensus tree in PAUP*4.0 (Swofford, 2002) using a Shimodaira-Hasegawa (SH) likelihood test with 1,000 bootstrap replicates and full optimization to test alternative hypotheses.

Figure 3.2
Alignment of partial *slo1* nucleotide sequence from species examined. This alignment was used for phylogenetic analyses and is also available in .fas format. This partial sequence contains both the pore region and sixth membrane-spanning domain (S6) involved in channel selectivity. Exons are labeled after Beisel *et al.* 2007 starting with exon 9. Note that according to this exon numbering system, exon 11 is alternatively spliced in place of exon 10 and is thus skipped in this alignment as the transcript containing exon 10 appears to predominate both in the species examined in this study and throughout the literature. Sequences are listed by genus name in phylogenetic order after Nelson 2006.

Figure 3.2 (continued)

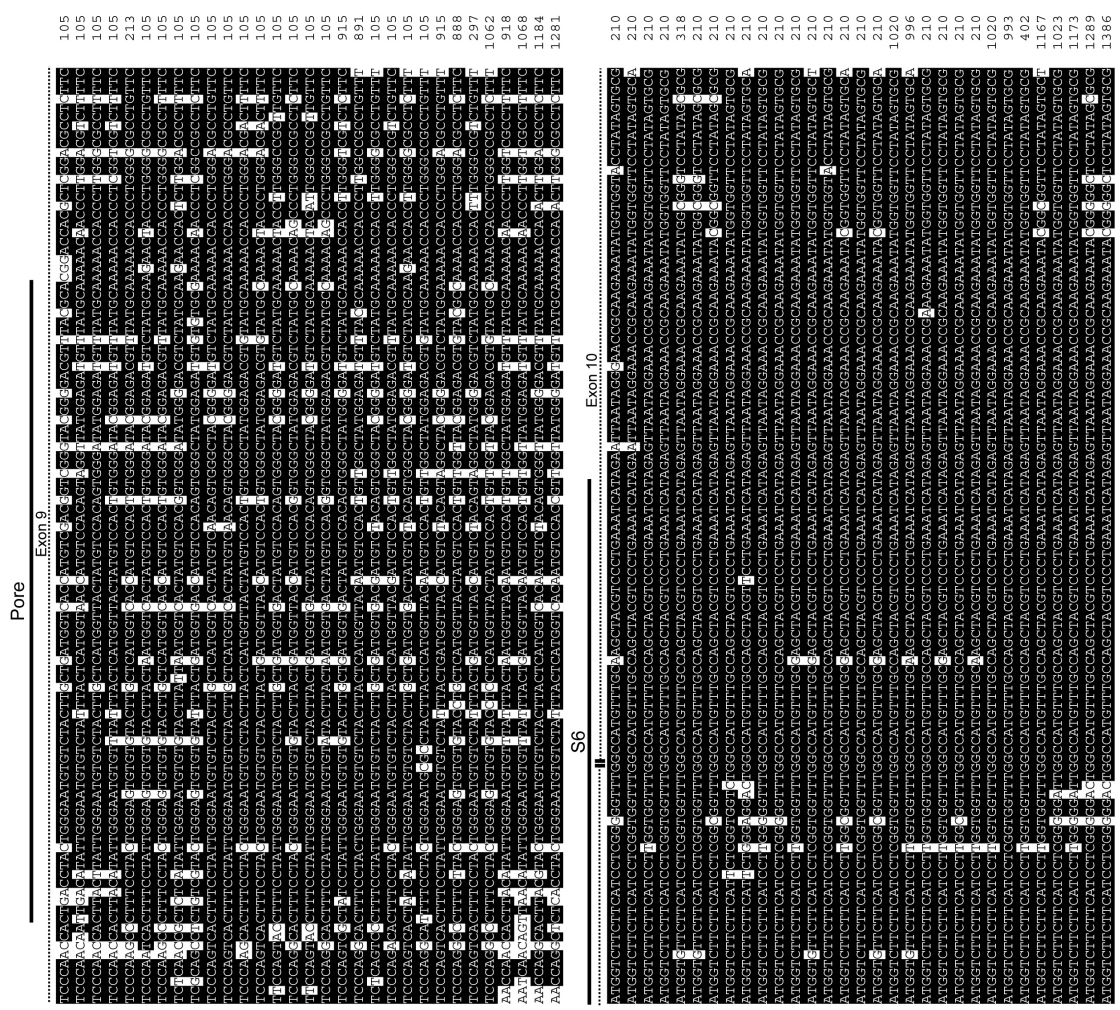


Figure 3.2 (continued)

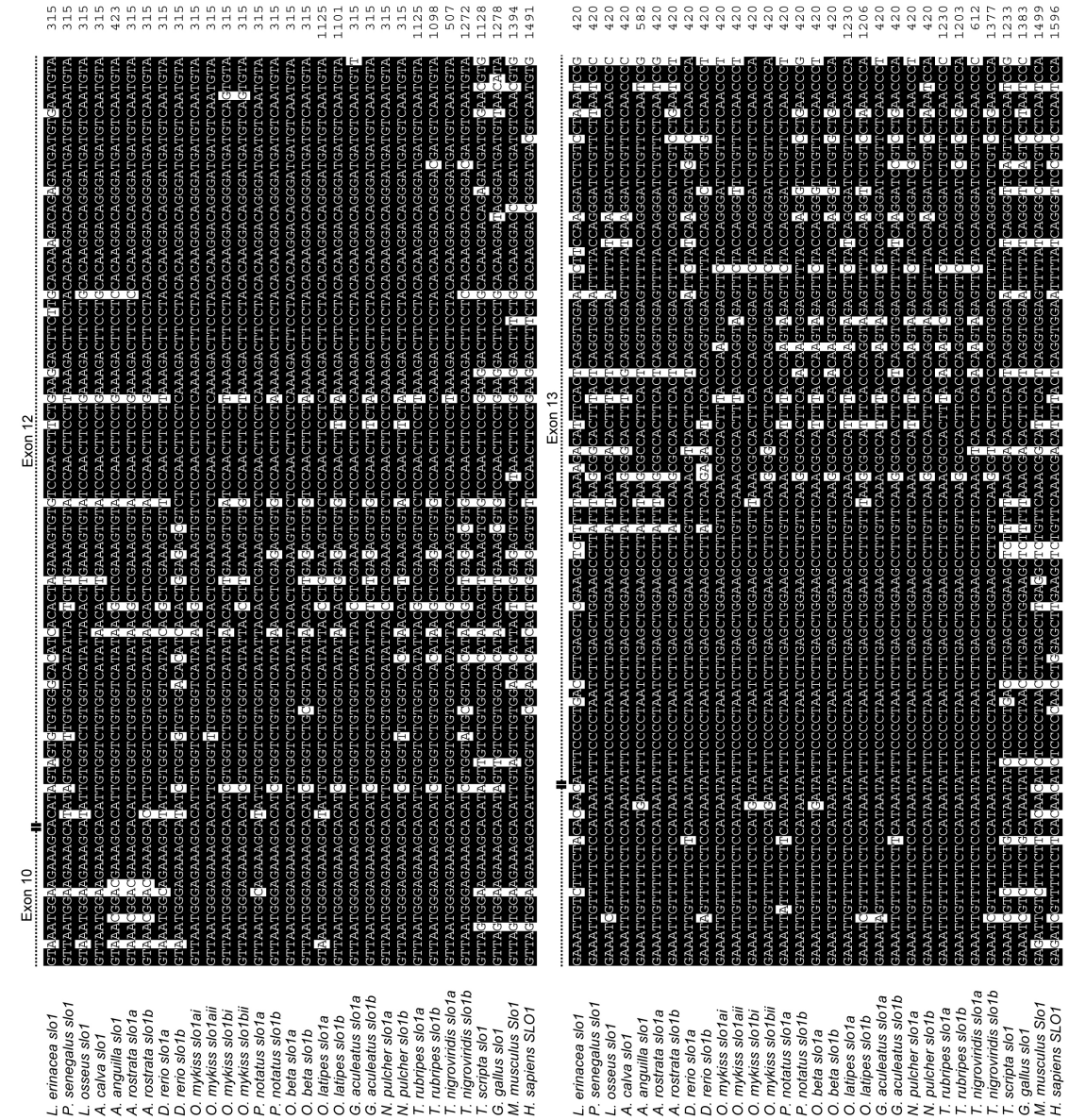
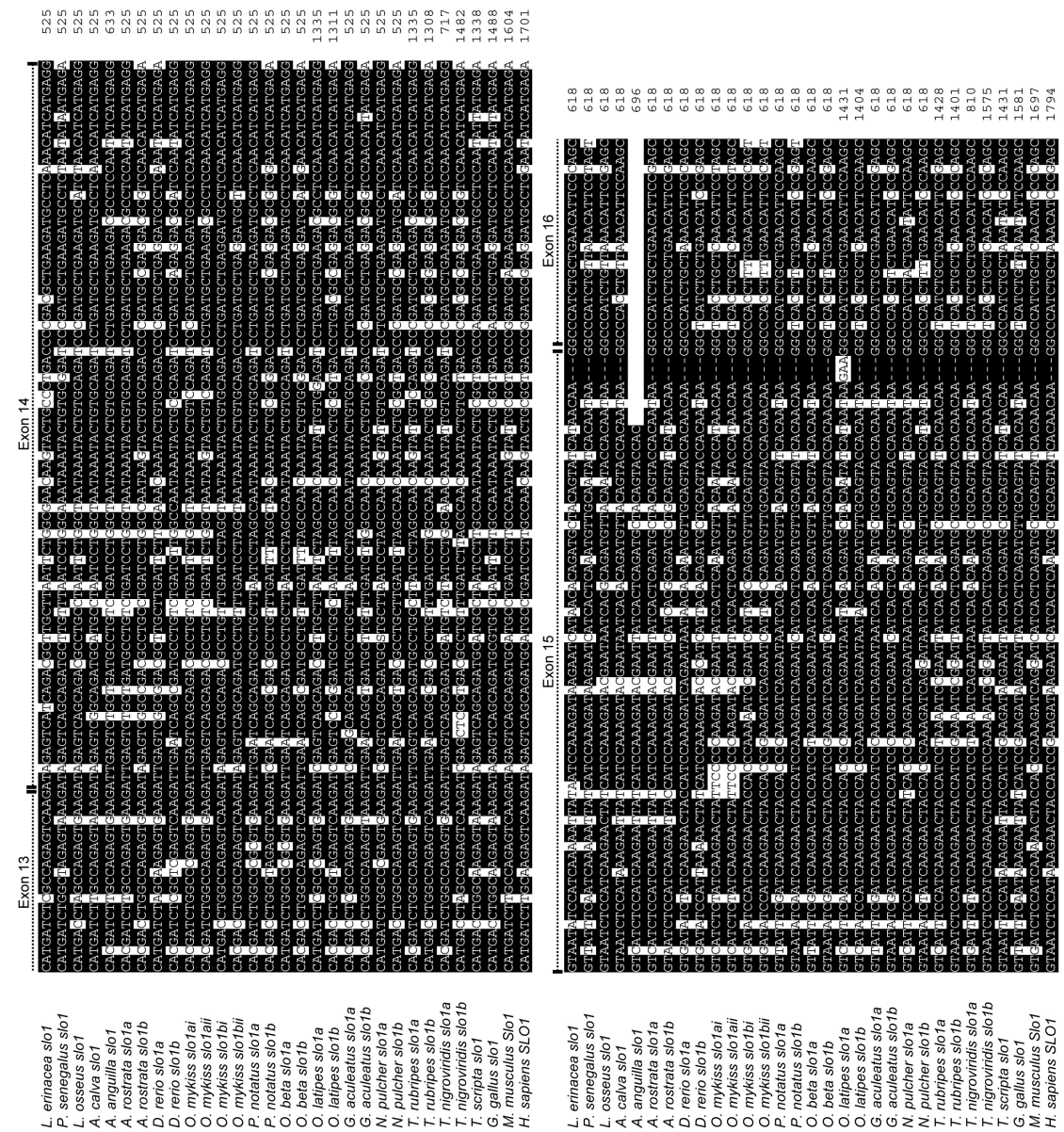


Figure 3.2 (continued)



Relative Evolutionary Rate Analysis

The Tajima (1993) relative rate test was conducted using MEGA4 (Tamura et al., 2007) for *slo1* paralogues with *L. erinacea* as an outgroup for the predicted 206 amino acid long protein sequences corresponding to the 618 bp sequence obtained above. Additionally, the complete amino acid sequences for the four teleost duplicate pairs available from Ensemble were compared with *H. sapiens SLO1* as an outgroup.

Alternative Splicing Analysis

Genomic sequences were retrieved from Ensembl as of July 2008. Ensembl combines information on species-specific protein sequences, protein sequences from closely related species, and cDNA and EST sequences to produce annotated genomic sequences. Because of a lack of species-specific as well as closely related species protein sequences, the majority of supporting evidence used in Ensembl's construction of genomic sequences for teleost *slo1* genes appears to have been based on tetrapod genomic sequences. Exon boundaries as identified in Ensembl were manually compared to exon boundaries in *M. musculus* and additional tetrapod sequences as needed (Beisel et al., 2007) to ensure that possible exons in teleost sequences were not excluded from the Ensembl sequences for lack of sequence similarity with tetrapod genes. Where alternatively spliced exons present in tetrapods where not labeled in Ensembl, unlabeled exons were identified by translation of the genomic sequence between previously identified exons.

Results

Two slo1 *Genes in Midshipman*

Two distinct *slo1* transcripts were first identified in the cDNA from CNS and the sensory epithelium of the auditory saccule using PCR with degenerate primers (figure 3.2, see table 3.2 for GENBank accession numbers). Additional degenerate primers were used to expand upon the initial sequence obtained in order to gather additional sequence information from which to produce probes for genomic southern blots which differed enough in sequence to prevent cross-reactivity between the two sequences. Genomic southern blots (figure 3.3) confirmed that the two *slo1* transcripts identified in *P. notatus* were the product of two genes. It was unlikely that one of the two very similar *slo1* transcripts identified was a product of another member of the *slo* gene family because of the high level of sequence divergence between *slo1* and *slo2* and the lack of expression of *slo3* in the brain of mammals (Salkoff et al., 2006).

The expression of *slo1a* and *slo1b* was examined in both neural and non-neural tissues from adult male *P. notatus* using RT-PCR. PCR revealed that both *slo1a* and *slo1b* appeared to be expressed in the CNS, auditory (saccular) epithelium, heart, and pituitary (figure 3.4A). Only *slo1a* was expressed in both trunk and vocal muscle, while in the gill and intestine only *slo1b* was expressed (figure 3.4A). Control digests of pure *slo1a* and pure *slo1b* confirmed that the digest conditions were sufficient to completely digest all *slo1b* while leaving *slo1a* intact (figure 3.4B). Together, the results suggested that *slo1* expression patterns differed between skeletal (trunk and vocal), smooth (gill and intestine), and cardiac (heart) muscle types which express *slo1a*, *slo1b*, and both *slo1*

Table 3.2
ENSEMBL database entries of teleosts for which *slo1* exons are present in tetrapods but are absent or truncated in teleosts.

Organism	Gene	Ensembl Gene	Absent Exons (Alternative Splice Sites) Based on Mouse Nomenclature
Danio rerio	slo1a	No Ensemble Entry	--
	slo1b	ENSDARG00000060237	4 (1)[a]
Oryzias latipes	slo1a	ENSORLG00000006622	4 (1) 19 (3)[b]
	slo1b	ENSORLG00000008798	4 (1) 19 (3)
Gasterosteus aculeatus	slo1a	ENSGACG00000008829	4 (1) 19 (3)[b] 29 (6)[c]
	slo1b	ENSGACG00000002248	4 (1) 19 (3)
Takifugu rubripes	slo1a	ENSTRUG00000003720	4 (1) 19 (3)[b]
	slo1b	ENSTRUG00000014048	4 (1) 19 (3)
Tetraodon nigroviridis	slo1a	ENSTNIG00000008628	4 (1) 19 (3)[b]
	slo1b	ENSTNIG00000013160	4 (1) 19 (3)

[a] Ensembl entry ends with exon 17.
[b] Conserved 5' SRKR amino acid sequence with potential species-specific 3' end.
[c] Potential partial/truncated exon present.

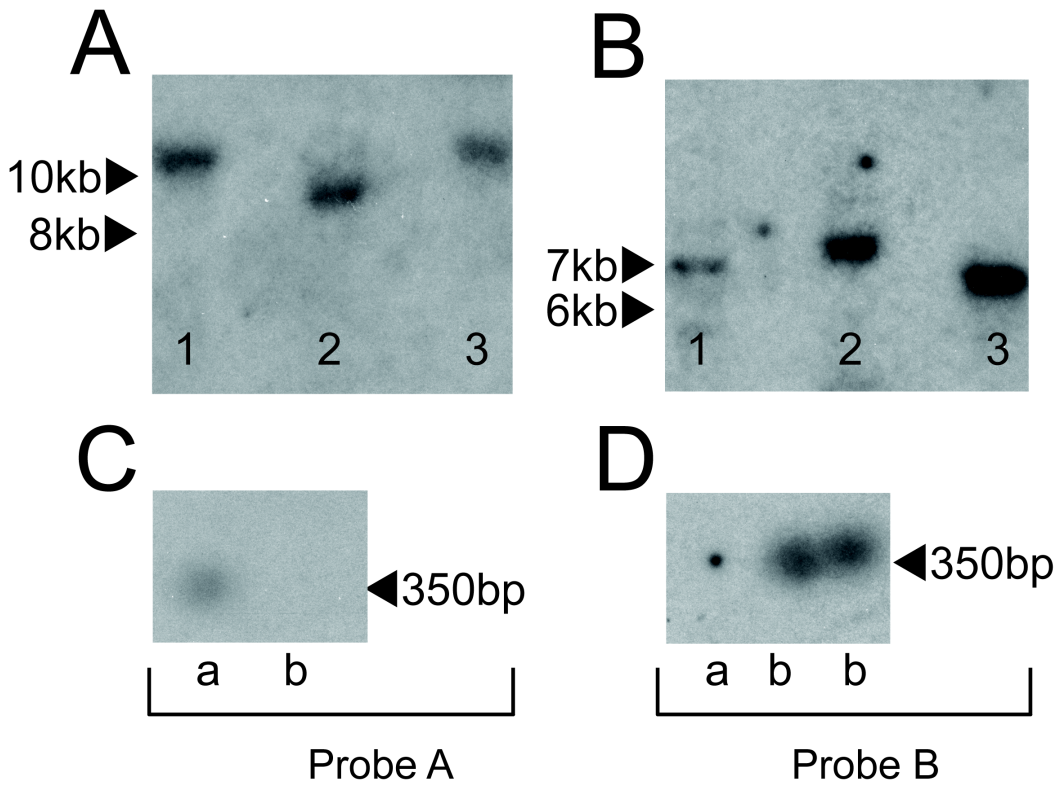


Figure 3.3
Southern blots of the midshipman fish (*P. notatus*) liver genomic DNA incubated with P32-labeled probes to *slo1a* (A) and *slo1b* (B) from homologous regions of the two paralogues. Genomic DNA was digested with *BamHI* (1), *EcoRI* (2), and *HindIII* (3) resulting in bands of different sizes between genes confirming that *P. notatus slo1a* and *slo1b* are the products of two genes. (C,D) Each probe was tested for cross-reactivity between *slo1* paralogues by probing the ~350 bp template sequence for the *slo1a* (a) and *slo1b* (b) probes. Both probes reacted only with its probe template and failed to label the template for the paralogue confirming the specificity of the probes used in the above panels (A,B).

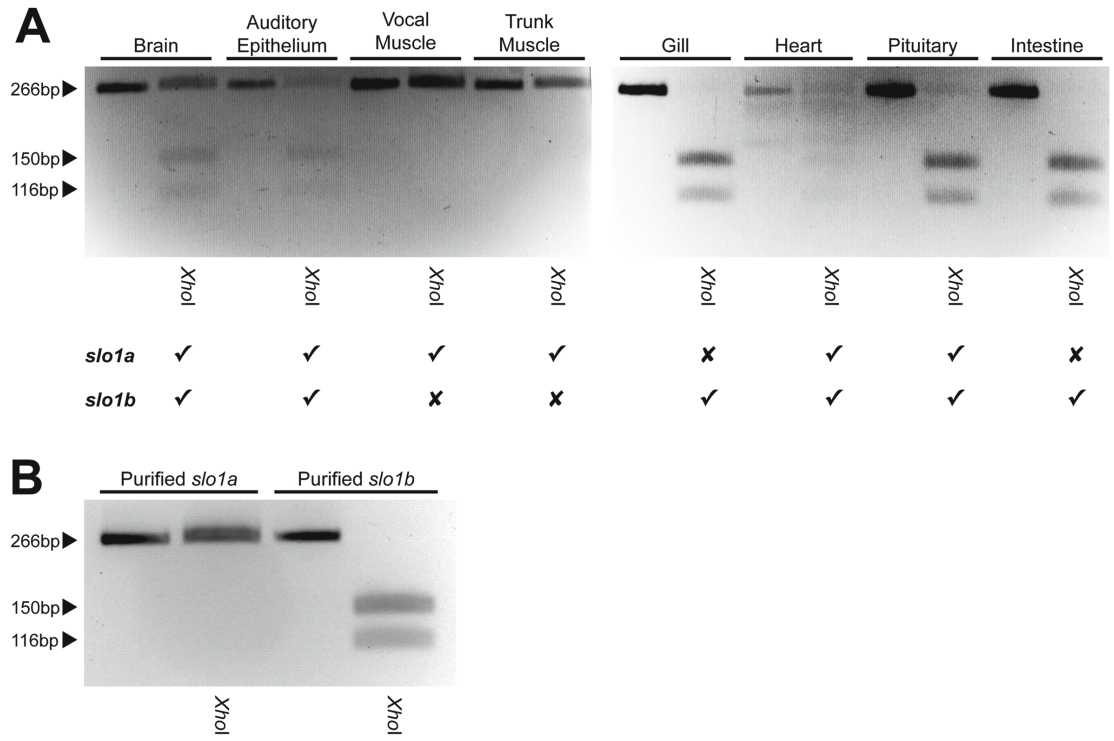


Figure 3.4
Relative expression of *slo1a* and *slo1b* within several tissues from the midshipman fish *Porichthys notatus*. Using primers designed to regions of both *P. notatus slo1* genes with 100% nucleotide identify, a 266 bp fragment of both *slo1* transcripts was amplified using PCR on (A) cDNA reverse transcribed from brain (brain and anterior spinal cord), auditory (saccular) epithelium, vocal muscle, trunk muscle, gill, heart, pituitary and intestine. Half of each purified PCR reaction (150-350 ng, depending on tissue type) was digested with *XhoI* that digests only *slo1b* (150 and 116 bp pieces) in *P. notatus* as *slo1a* lacks a *XhoI* recognition site. Undigested PCR product was run on a 3% agarose gel alongside *XhoI* digested samples to examine the relative expression of *slo1a* and *slo1b* within each tissue. Both vocal and trunk muscles express only *slo1a* (100% undigested by *XhoI*) while gill and intestine express only *slo1b* (100% digested by *XhoI*). The remaining tissues expressed a mixture of both *slo1a* and *slo1b*. The expression of *slo1a* and *slo1b* is noted below the gel for each tissue. (B) The same PCR reaction was conducted on sequenced plasmids containing either *P. notatus slo1a* or *slo1b* to produce samples that were either pure *slo1a* or pure *slo1b*. 400ng of each purified PCR product was digested with *XhoI* under the same reaction conditions as used in panel A. As in A, each digest was run on an agarose gel alongside an equal amount of undigested PCR product. These digests confirm that the reaction conditions used on all samples are sufficient to digest 100% of the purified *slo1b* sample while leaving *slo1a* intact.

genes, respectively. Sequencing the PCR products from muscle and gill confirmed the presence of only *slo1a* in trunk and vocal muscle and only *slo1b* in gill. Sequencing also revealed differences in splicing between the paralogues within these peripheral tissues where *slo1a* transcripts contained exon 10, as was the case with all *slo1* transcripts sequenced from the CNS of all teleosts examined, while *slo1b* transcripts contained exon 11 (see figure 3.5 for splicing pattern). Because we did not examine *slo1* expression in non-CNS tissues in other species, we do not know if the differences in expression of exon 10 versus exon 11 in peripheral tissues were unique to *P. notatus*. Exons 10 and 11 are mutually exclusive in the final vertebrate *slo1* transcript (Beisel et al. 2007), with exon 10 present in the majority of published transcript sequences from tetrapods.

In sum, two *slo1* genes were identified in the CNS and peripheral tissues of *P. notatus* with some peripheral tissues showing expression of only one paralogue, with paralogues differing in alternative splicing patterns of potential importance to channel physiology. Exon 11 encodes a protein that lacks one of two glutamic acid residues present in exon 10 which provide electrostatic forces responsible for the large K^+ current through BK channels (Brelidze et al., 2003; Nimigean et al., 2003).

Two slo1 *Genes in Teleosts*

The surprising discovery of two *slo1* genes raised the question of whether this duplication was unique to *P. notatus* and, if not, when the duplication event occurred. We used the Ensembl genome browser to first obtain *slo1* sequences of other teleosts including *G. aculeatus*, *O. latipes*, *T. nigroviridis*, and *T. rubripes*, all of which

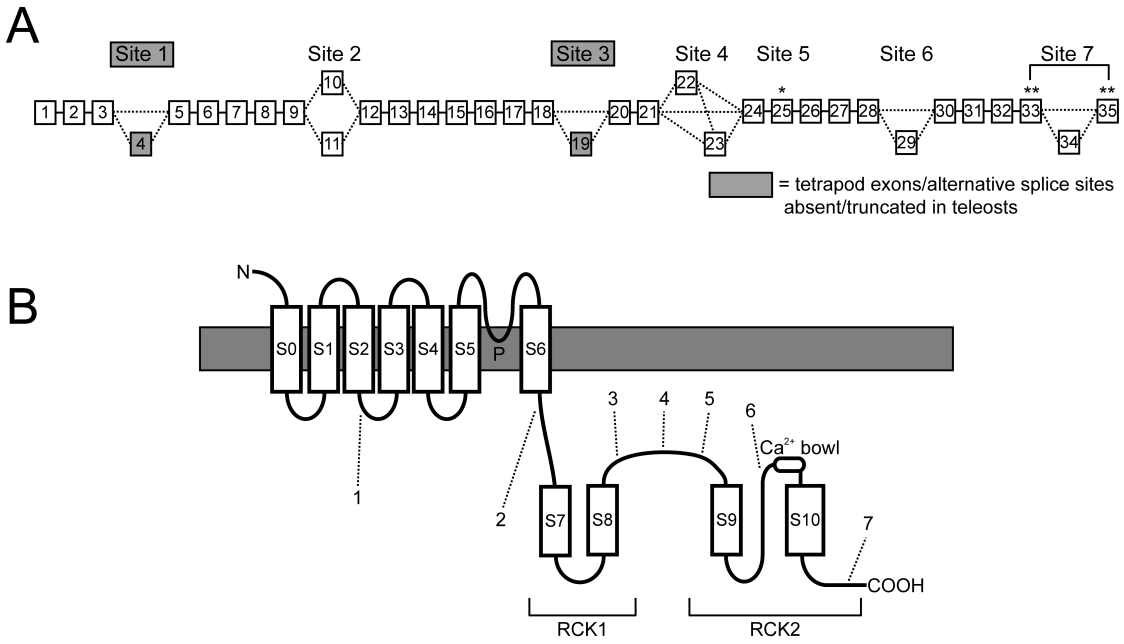


Figure 3.5
(A) Patterns of alternative splicing in tetrapods and teleosts as seen in the organization of exons into a *slo1* transcript. Sites of alternative splicing are labeled with dotted lines showing the ways in which alternatively spliced exons are included/excluded from the final transcript. Those sites present in tetrapods but absent/truncated in teleosts are shaded in grey. Asterisk (*) marks exon with variable open reading frame. Asterisks (**) mark possible stop codons. All exon and splice site numbers are after Beisel et al. 2007. (B) A representation of the membrane topology of the protein encoded by the *slo1* gene of vertebrates with sites of alternative splicing indicated. The location of the membrane spanning domains (S0-S6), pore (P), intracellular hydrophobic domains (S7-S10), two regulator of potassium conductance (RCK) domains, and calcium bowl are labeled (adapted from Beisel et al. 2007).

possessed two *slo1* genes (table 3.2). Duplicate *slo1* genes are each located on separate chromosomes in *G. aculeatus* (chromosomes 5 and 6), *O. latipes* (chromosomes 15 and 19), and *T. nigroviridis* (chromosomes 2 and 17); comparable data are unavailable in the current genome assembly for *T. rubripes*. For those species for which the chromosome location of *slo1a* and *slo1b* are known, both genes are flanked by some of the same genes including annexin A11 (ANXA11), zinc finger CCHC domain-containing protein 24 (ZCCHC24), and zinc finger protein 503 (ZNF503). The conserved synteny between *slo1* duplicates in these species suggests *slo1* duplication was the result of a teleost whole genome duplication event (Amores et al. 1998; Taylor et al. 2001). To further test this hypothesis, we examined basal teleosts and outgroups to the teleost whole genome duplication event.

The same degenerate primers used to obtain both *slo1* gene transcripts from *P. notatus* were used in PCR on whole CNS cDNA from other teleosts (*A. rostrata, D. rerio*, *O. mykiss*, *O. beta*, *G. aculeatus*, *N. pulcher*); non-teleost basal actinopterygians (*P. senegalus, L. osseus*, *A. calva*); and a chondrichthyan (*L. erinacea*). For all species examined, PCR produced a single band of the predicted size. When PCR products were subcloned and sequenced (table 3.2), it was revealed that while basal actinopterygians and chondrichthyans expressed only a single *slo1* transcript, all teleosts examined expressed two *slo1* transcripts similar to the two *slo1* genes identified above in both nucleotide (figure 3.2) and predicted protein sequence alignments (figure 3.1). The latter included *D. rerio,* despite the observation that the Ensembl database contained only one incomplete *slo1* entry (table 3.1). Unlike the above species, *O. mykiss* expressed four *slo1* transcripts. *Slo1* paralogues showed high NT and AA sequence identity (84.1% *D.*

rerio - 92.7% *A. rostrata*, 97.6% *P. notatus* - 99% multiple species, respectively). Both NT and AA sequence identity between teleost *slo1* duplicates and *H. sapiens SLO1* were similar between paralogues, differing by only 1-2% (81.7% *P. notatus slo1a* - 84.8 *D. rerio*, 97.1% many species - 98.5 *O. latipes, T. rubripes*, respectively). Similarly high protein sequence identity was observed between the full-length protein sequences of teleost *slo1a* and *slo1b* in Ensembl (86.0-89.8%).

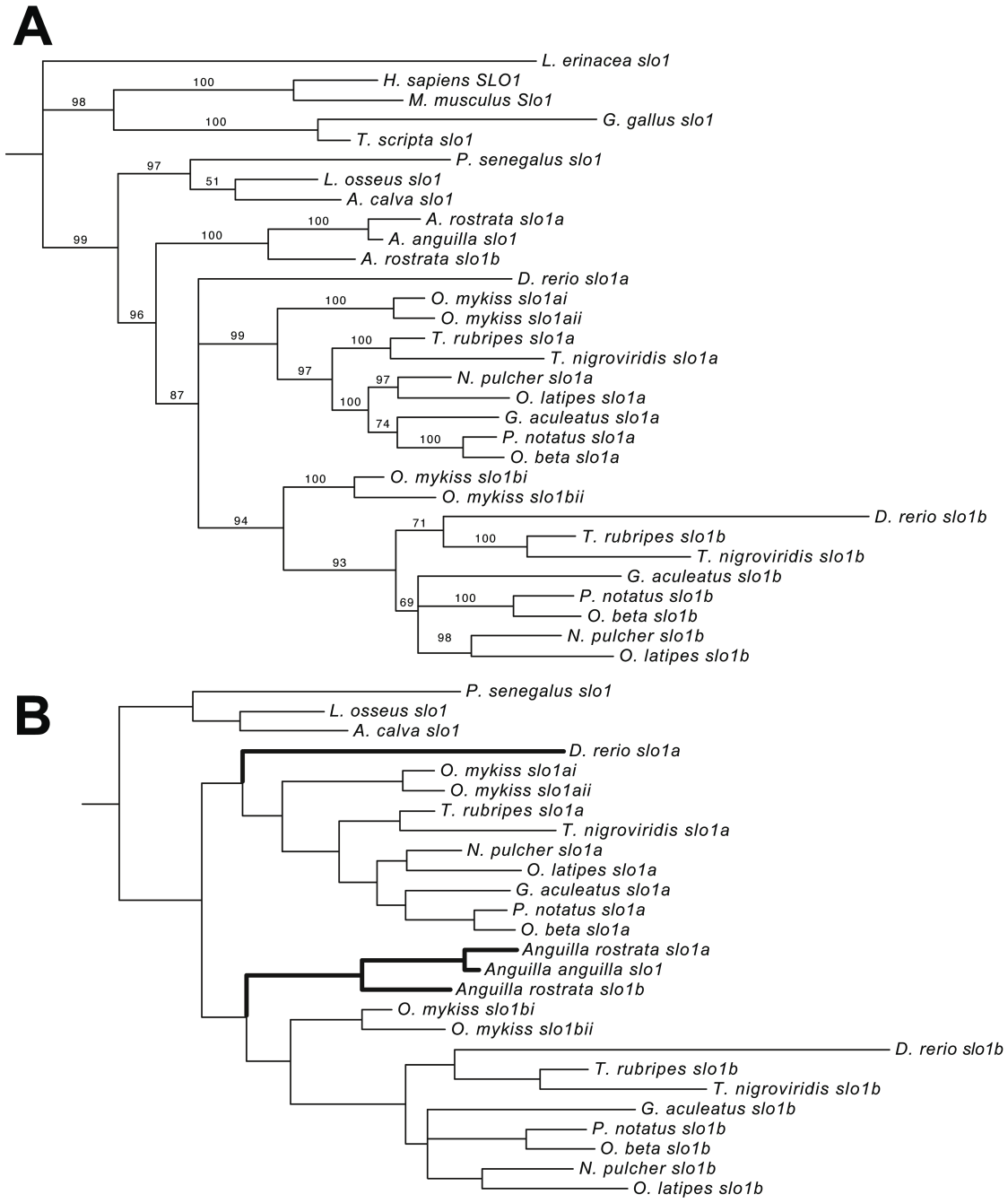
Together, our data demonstrate the duplication of the *slo1* gene and retention of both copies in teleosts. The teleosts we examined had high sequence identity between *slo1* duplicates over their length and they both share the same highly conserved pore region of the BK channel found in tetrapods.

Multiple slo1 *Gene Duplication Events*

The nucleotide sequences of *slo1* genes from 19 species including 11 teleosts were aligned and a phylogenetic analysis was conducted to determine the relationship between tetrapod *slo1* genes and duplicate teleost *slo1* genes. The entire sequence was used as compared to the 618 bp region acquired through PCR for those species for which the entire coding sequence was available through sequence databases. This may help explain some of the long branch lengths in those taxa for which there were more characters included in the analysis. A monophyletic group of tetrapod/ sarcopterygian *slo1* genes was recovered as a sister to the monophyletic actinopterygian *slo1* genes (figure 3.6A). Note that only a single *slo1* sequence appears for *A. anguilla* reported by Lionetto *et al.*(2008). As reported above, we identified two *slo1* transcripts in the brain of the cogener *A. rostrata*. Placement of *A. anguilla slo1* as a sister to *A. rostrata*

Figure 3.6
(A) Bayesian estimate of phylogeny for vertebrate *slo1* genes. Posterior probabilities are indicated above each branch. The Ensembl and GenBank numbers for each sequence can be found in the methods section and supplementary table 1. (B) A portion of a tree modified (in bold) from the Baseyian consensus tree (A) in order to resolve the polytomy between *Danio rerio slo1a* and the *slo1a* genes from all other more derived teleosts. An SH test confirmed no significant difference (p=0.106) between the two trees and that the tree in the lower panel (B) has a more favorable likelihood score. *Slo1* genes of the genes in *Anguilla rostrata* have been relocated (in bold) as paralogues of *slo1b* of other teleosts, a move favored by likelihood over the Bayesian estimate (p = 0.482, SH test). The results indicate a teleost whole genome duplication event was followed by loss of *slo1a* in anguilliforms and that a second independent duplication event led to both *slo1* paralogues present in *A. rostrata*.

Figure 3.6 (continued)



slo1a suggests that either *slo1b* was lost in *A. anguilla* or that this paralogue is not expressed in the intestinal epithelium in this animal (see discussion).

The Bayesian estimate of phylogeny (figure 3.6A) supported three duplication events within the teleost lineage, the first occurring within the anguilliforms, the true eels. A second duplication event, possibly of the whole teleost genome, occurred prior to the ostariophysans that include the zebrafish *D. rerio*. A third round of duplication, likely autotetraploidization, occurred in salmonids, represented here by *O. mykiss* (this event is referred to as autotetraploidization instead of "whole genome duplication" because diploidization of the salmonid genome is apparently incomplete (Allendorf and Thorgaard, 1984; Phillips et al., 2006).

Resolving the polytomy of *D. rerio slo1a* as a sister clade to the other ostariophysan *slo1a* genes (figure 3.6B bold) resulted in a tree that was not significantly different from the Bayesian consensus tree (SH test, $p = 0.106$), with a more favorable likelihood score. A second SH test between the Bayesian consensus and a tree based on the hypothesis of a single *slo1* duplication event at the origin of teleosts followed by a second duplication event in salmonids resulted in a significant difference between the trees ($p = 0.041$) with likelihood favoring the consensus tree. As a result, we conclude that the duplication of *slo1* in anguilliforms is a third duplication event independent from those in other teleosts and the salmonid lineage. Thus, additional SH tests were conducted to determine the timing of the anguilliform *slo1* duplication relative to teleost whole genome duplication.

We tested the hypothesis that *A. rostrata slo1* paralogues were the result of an independent gene loss and subsequent duplication event in anguilliforms following the

divergence of *slo1a* and *slo1b* in other teleosts. Several previous studies have provided evidence for independent gene loss in elopomorphs, occasionally followed by a secondary duplication (Hoegg et al. 2004, de Souza et al. 2005, Crow et al. 2006, Hurley et al. 2007). SH tests could reject neither the hypothesis that anguilliform *slo1* genes are associated with teleost *slo1a* ($p = 0.223$) or *slo1b* ($p = 0.482$) nor could they distinguish between either hypothesis ($p = 0.144$); however, grouping anguilliform *slo1* sequences with *slo1b* of other teleosts had the best likelihood score of the competing tree topologies. The analysis supported the hypothesis of a loss of one of the *slo1* duplicates produced by whole genome duplication followed by a secondary duplication of the remaining *slo1* gene in *A. rostrata* (figure 3.6B), a pattern observed in other anguilliform genes and consistent with the proposed timing of the teleost whole genome duplication event (see discussion).

In sum, the phylogenetic analyses identified a single *slo* duplication event at the origin of teleosts followed by an independent loss and secondary *slo1* duplication event in anguilliforms, and a third, independent *slo1* duplication in salmonids.

slo1 *Paralogues Have Evolved at Similar Rates*

Tajima (1993) relative rate tests were conducted to determine if either *slo1* paralogue evolved at an accelerated rate following duplication. There were no significant differences in relative rate of evolution between any teleost *slo1* paralogues within the 206AA long sequence, corresponding to the pore and S6 domains, obtained for all species with the chondrichthyan *R. erinacea slo1* serving as an outgroup. Examination of the entire protein coding sequences from the teleosts *G. aculeatus*, *O. latipes*, *T. rubripes*,

T. nigroviridis with *Homo SLO1* as an outgroup also resulted in no significant differences in relative rates of evolution between teleost *slo1* duplicate pairs with sequence identities between 78.1% and 81.8% at the nucleotide level.

Teleost slo1 *Genes Contain Fewer Sites of Alternative Splicing than Tetrapods*

Rates of alternative splicing are inversely correlated with gene family size (Kopelman et al., 2005). Thus, we examined the duplicate *slo1* genes of teleosts for evidence of reduced alternative splicing relative to the single *slo1* gene in tetrapods. The genomic sequences of *slo1* genes available from Ensembl for five teleosts (*D. rerio*, *O, latipes*, *G. aculeatus*, *T. rubripes*, and *T. nigroviridis*) were compared to the genomic sequence and splice sites of the mouse (*M. musculus*) *Slo1* using the mouse numbering system (Beisel *et al.* 2007). Note that our analysis of *slo1* genomic sequences in *D. rerio* was limited by the presence of only a single Ensembl entry that terminates at exon 17 (out of the total 35). Table 3.1 and figure 3.5 provide an overview of the analysis as well as a diagrammatic representation of alternative splicing in *slo1* genes.

All exons involved in alternative splicing at sites 2, 4, 5, 6, and 7 were found to be present in both duplicate *slo1* genes in the teleosts examined. In all the teleosts examined, we found evidence for loss of the exons at splice sites 1 and 3 in either both *slo1* co-orthologues (site 1) or in *slo1b* alone (site 3). Splicing in both sites is characterized by the insertion of none, part, or all of a non-constitutively expressed exon (see figure 3.5).

Exon 4 (splice site 1) in mouse was absent from both *slo1* duplicates in all five teleosts examined, including *D. rerio*. This is consistent with the findings of Beisel *et al.* (2007), who reported difficulty in identifying exon 4 in *D. rerio*. Excepting the *D. rerio*

sequence that is truncated at exon 17, the teleost sequences corresponding to exon 19 in mouse (splice site 3) were highly divergent 3' of the conserved initial SRKR amino acid segment in *slo1a*, while the entire exon was absent in *slo1b*. Beisel *et al.* (2007) noted that the 3' end of exon 19, which is truncated in some tetrapod splice variants, appears to be species-specific; this region has apparently diverged even further in teleost *slo1a*. The absence of even the highly conserved SRKR domain in *slo1b* suggests that exon 19 was differentially lost between *slo1a* and *slo1b* following duplication.

Lastly, we note that the only difference in either exon or splice site number between the *slo1* paralogues in teleosts was found in *G. aculeatus* in which exon 29 (splice site 6) was severely truncated at the 3' end. This truncated exon may, however, be the result of sequencing or alignment errors that may be resolved in new genome databases or through targeted genomic sequencing.

Discussion

As is the case with many genes and gene families in teleosts, we report a duplication of the *slo1* gene encoding the pore-forming α-subunit of BK channels in all of the species examined. Of the species in which we sequenced transcripts from cDNA, all expressed the full complement of *slo1* genes in the CNS, while all *slo1* paralogues possessed similar nucleotide and amino acid sequence identities with orthologous genes in tetrapods.

The partial *A. anguilla slo1* sequence obtained by Lionetto *et al.* (2008) overlaps with the region sequenced from the CNS in the present study, but came from cDNA reverse transcribed from RNA extracted from the intestine, a tissue which expresses only a single *slo1* duplicate in *P. notatus* (figure 3.4). The presence of two *slo1* genes in the

closely-related *A. rostrata* along with evidence of tissue-specific loss of expression of a *slo1* duplicate in *P. notatus* suggests that *A. anguilla* possesses two *slo1* genes, only one of which is expressed in intestine. The expression of four different *slo1* transcripts in the brain of *O. mykiss* is consistent with it and other salmonids having at least 13 *hox* clusters (Moghadam et al., 2005; Mungpakdee et al., 2008) as compared with seven in *D. rerio* (Amores et al., 1998). An autotetraploidization event in salmonids about 25-100mya (Allendorf and Thorgaard, 1984) accounts for the apparent second round of *slo1* duplication in *O. mykiss* following the teleost whole genome duplication event.

Whole Genome Duplication Events Across Teleosts

Bayesian estimate of phylogeny revealed relationships similar to what has been reported for actinopterygians (Nelson, 2006). Interestingly, polypteriforms, lepisosteiforms (gar), and amiiforms (bowfin) formed a monophyletic group that is a sister-group to teleosts, while both mitogenetic (Inoue et al., 2003) and nuclear genetic (Venkatesh et al., 2001) data have suggested polypteriforms are ancestral to the sister-group of teleosts including gars and bowfin. This difference between our analysis and previous studies may be due to the relatively small sequences used in our analysis compared to previous studies using multiple genes and morphological traits to determine the relatedness of these groups.

Duplication of *slo1* due to a teleost whole genome duplication event at the origin of teleosts (Hoegg et al., 2004), followed by autotetraploidization in salmonids, would predict only two duplication events. Phylogenetic analyses support this hypothesis, identifying a duplication event at the origin of teleosts, an independent loss and secondary duplication event following the divergence of the anguilliforms from the

teleost lineage, and a second round of duplication in salmonids. Crow *et al.* (2006) identified an independent duplication event of *hoxd4* in elopomorphs (*A. rostrata*, *Megalops atlanticus*) with all four *hoxd4* genes grouped with *hoxd4a* of other teleosts, thus mirroring the data in our study. The same study reported the duplication of *hoxa11* and *hoxb5* as a result of a single duplication event in the ancestor common to all teleosts, including elopomorphs (Crow et al., 2006)

Other studies utilizing nuclear gene sequences to time the whole genome duplication event in teleosts have reported only a single gene in elopomorphs whereas other teleosts possess two, including proopiomelanocortin gene (*POMC*) (de Souza et al., 2005), *sox11*, and *tyrosinase* (Hoegg et al., 2004; Hurley et al., 2007). In each case, the single elopomorph gene grouped onto a branch with one of the two teleost co-orthologues, suggesting that the teleost whole genome duplication event occurred prior to the divergence of elopomorphs and that gene loss following duplication was higher in elopomorphs than in other teleosts for these particular genes.

Ion Channel Duplication Events

Studies of ion channel gene duplication in several species of teleosts support the hypothesis that a whole genome duplication occurred prior to the divergence of basal teleosts (Amores et al., 1998; Taylor et al., 2001). This includes genes encoding the pore-forming alpha-subunits of voltage-gated sodium channels (*SCNA*) (Novak et al., 2006; Zakon et al., 2006), voltage-gated calcium channels (Wong et al., 2006), and shaker-related voltage-gated potassium channels (*KCNA*) (Few and Zakon, 2007; Hoegg and Meyer, 2007). Analysis of the shaker-related voltage-gated potassium channel gene

family (*KCNA*) showed that duplication of this family in the osteoglossomorph *Gnathonemus petersii* generally occurred as a result of the same event responsible for *KCNA* duplication in other teleosts (Hoegg and Meyer, 2007). By contrast, gene duplications in the voltage-gated sodium channel alpha-subunit gene family (*SCNA*) giving rise to three pairs of *scna* genes (*scn4a*, *scn5a*, and *scn8a*) in *G. petersii* were found to be independent from the duplication event in other teleosts (Novak et al., 2006). Novak *et al.* (2006) suggested these results could be due to artificial factors such as long-branch attraction; never the less, the results may reflect different patterns of gene duplication between teleosts and tetrapods in a given ion channel gene family. As is the case with several of the studies described above, the exact timing of gene duplication in basal teleosts sometimes suggests the presence of independent events which ultimately result in basal teleosts possessing a similar complement of duplicate genes as more derived species.

Our data is consistent with the previous studies described above and suggest that the original *slo1a* paralogue in anguilliforms produced by a teleost whole genome duplication was lost and the remaining paralogue duplicated in an independent event. Additional analyses are needed to determine whether the instances of gene loss and duplication observed in basal teleosts following teleost whole genome duplication occurred either in a common ancestor of basal teleosts or were the result of a series of smaller, lineage-specific events. Because the sequence data from anguilliforms used in the current study is from the highly conserved pore domain, it may explain the inability to more definitely resolve the phylogenetic relationship between the *slo1* genes of anguilliforms and other teleosts. Further sequencing of the *slo1* genes of anguilliforms to

include less highly conserved domains may help resolve this relationship more concretely.

Comparisons with Tetrapods and Invertebrates

Analysis of the genomic sequences of the teleosts *O. latipes*, *G. aculeatus*, *T. rubripes*, and *T. nigroviridis* reveals a strict conservation of constitutive exons between both *slo1* co-orthologues that extends to the single *slo1* gene found in tetrapods and invertebrates (Fodor and Aldrich, 2008). The presence of twice the complement of *slo1* genes in teleosts has apparently not relaxed the constraints on constitutive exons. By contrast, alternatively spliced *slo1* exons are not as highly conserved between tetrapods and teleosts where exon 1 is lost in both *slo1* co-orthologues and exon 19 is lost in *slo1b*. Thus, unlike constitutive exons, *slo1* duplication in teleosts may have relaxed constraints on alternatively spliced exons. The retention in *slo1a*, but loss in *slo1b*, of exon 19 is evidence of a relaxed constraint that may have facilitated the retention of these duplicate genes via subfunctionalization (Hughes, 1994; Force et al., 1999; Postlethwait et al., 2004).

The presence of at least two *slo1* genes in teleosts may achieve the same transcript diversity as the single *slo1* gene found in both tetrapods and invertebrates, which produce transcript diversity via more extensive alternative splicing. The question of whether tetrapod-specific splice sites are due to a gain in tetrapods versus a loss in teleosts requires investigating the genomic sequence of *slo1* in a common vertebrate ancestor. Comparison with invertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans* in which *slo1* expression and physiology has been extensively studied is not

informative in this regard as shared patterns of alternative splicing between these invertebrates and tetrapods are the result of convergent evolution (Fodor and Aldrich, 2008). The study of *slo1* sequences in either ancestral chordates (tunicates, lancelets) or craniates (hagfishes) might address the question of splicing gain versus loss in tetrapods as compared to teleosts.

Two slo1 *Genes in Vocal Midshipman Fish*

Differential expression patterns between *slo1* paralogues in *P. notatus*, including divergent splicing patterns between paralogues in peripheral tissues (gill, trunk and vocal muscle), suggests differences between duplicates in *cis*-regulatory elements controlling tissue-specific expression and splicing of each gene. As is the case with the inclusion of exon 11 instead of exon 10 in *P. notatus slo1b* in gill (vascular smooth muscle), such differences between paralogues may code for ion channels with different functional properties. The exchange of exon 10 for exon 11 results in a change in several residues in this 31AA long sequence including a substitution of an alanine residue for the second glutamic acid residue in the PEIIE domain labeled by an asterisk in figure 1. These glutamic acid residues form a ring of eight negative charges (two from each α-subunit in the BK-channel tetrameric structure) at the entrance to the channel's intracellular vestibule, increasing K^+ concentrations within the vestibule via electrostatic interactions resulting in the large K^+ conductance for which BK channels are named. Reducing the amount of negative charge within this domain by mutating either or both glutamic acid residues has been shown to decrease single-channel current amplitude (Brelidze et al., 2003; Nimigean et al., 2003). A change to the alternative exon at the same site in

Drosophila has been shown to produce a BK channel with decreased single channel conductance, decreased open probability, decreased calcium sensitivity, and faster kinetics (Lagrutta et al., 1994). Together, the data suggest that *P. notatus slo1a* and *slo1b* have different mechanisms controlling their expression across tissue types and that alternative splicing of these paralogues differs in such a way as to produce channels which likely differ in their physiological properties. Further investigation of the promoter regions of these paralogues is necessary to determine the extent of these differences including whether either or both genes share the multiple promoters and estrogen-responsive elements which regulate transcription of *M. musculus Slo1* (Kundu et al., 2007).

Subfunctionalization of *slo1* genes between different muscle types may reflect the varying roles of BK channels in tissue types with radically different functional demands. The role of BK channels in vascular smooth muscle, such as that found in the gill, in maintaining blood pressure has been studied extensively (Salkoff et al., 2006). The demands of smooth muscle are much different from the vocal muscle of toadfish, including *P. notatus* and *O. beta*, which is among the fastest contracting vertebrate muscles (Rome, 2006). Because toadfish vocal muscles have the fastest Ca^{2+} transients measured in any muscle (Rome, 2006), BK channels in these tissues presumably need to be much more sensitive and/or rapid in their response to Ca^{2+} than BK channels in other tissues. *P. notatus slo1a*, the only *slo1* gene expressed in vocal muscle, may encode a channel with such differences compared to *slo1b*. In this scenario, following duplication of *slo1* genes in teleosts, the physiological demands for rapid contraction in vocal muscle may have selected against the expression of *slo1b* that likely encodes a channel with

reduced conductance/slower kinetics than *slo1a* (see above). Alternatively, *slo1a* may be post-translationally modified in a way as to confer enhanced Ca^{2+} activation such as via interaction with the β1 subunit which enhances Ca^{2+} activation (McManus et al., 1995) and is expressed in skeletal muscle in mammals (Jiang et al., 1999).

To our knowledge, this study is the first to examine the expression of the gene(s) encoding BK channels in the auditory division of the inner ear (saccule) of a teleost fish. Unlike all other vertebrates studied previously, BK channels in the midshipman saccule are encoded by two *slo1* genes. Midshipman fish thus provide a unique opportunity to investigate how molecular plasticity, in this case the expression of BK channels, may contribute to the diversity in electrical tuning of the peripheral auditory system observed for tetrapods with auditory ranges overlapping that of midshipman (Fettiplace and Fuchs, 1999). *Slo1* duplication may also contribute to the steroid-dependent shifts in frequency encoding by the midshipman saccule that enhance the detection of male advertisement calls (Sisneros et al., 2004). Studies of mammals have already shown that the expression and alternative splicing of *slo1* are regulated by steroid hormones (Zhu et al., 2005; Kundu et al., 2007).

Concluding Comments

The frequency of alternative splicing is inversely correlated with gene family size (Kopelman et al., 2005). Consistent with this pattern, alternative splicing decreases in paralogues following gene duplication (Su et al., 2006). While teleosts may have reduced alternative splicing within each *slo1* co-orthologue compared to tetrapods, they may achieve similar levels of protein diversity through the combination of two *slo1* genes. *In*

vitro expression studies are required to determine whether heteromeric channels can be assembled by combining transcripts of both *slo1* co-orthologues. The *mitf* gene family, in which two teleost genes are homologous to two isoforms of a single mammalian gene (Lister et al., 2001; Altschmied et al., 2002), is exemplary of how these two mechanisms can be used interchangeably to achieve comparable levels of protein diversity as well as how subfunctionalization can preserve duplicate genes.

The conserved senteny between duplicate genes, together with the reduction of alternative splicing compared to tetrapods, support the hypothesis of a whole genome duplication event followed by retention of duplicate genes via the subfunctionalization model (Hughes, 1994; Force et al., 1999; Postlethwait et al., 2004). This model is further supported by the differential expression patterns of *slo1* paralogues in midshipman fish. *In vitro* expression studies using patch clamp to characterize the physiological properties of the BK channels produced from *slo1* paralogues will be necessary to determine the subdivision of functions between the duplicate genes in what accounts for approximately half of all living vertebrate species.

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CHAPTER 4

BK_{Ca} CHANNEL EXPRESSION MEDIATES AUDITORY HAIR CELL PLASTICITY

Abstract

From opsin and visual pigment expression in photoreceptors to G-protein coupled olfactory receptors in olfactory receptor neurons, genetic profiles define the limits of sensory detection. Here, we demonstrate that abundance of a gene (*slo1a*) encoding the pore-forming α-subunit of the large-conductance calcium-activated potassium (BK_{Ca}) channel in the auditory sensory epithelium (saccule) of a vocal fish parallels seasonal plasticity in frequency encoding at the hair cell level. *slo1a* abundance is greatest in reproductive animals when hair cell thresholds are lowest, especially over the frequency range of natural call upper harmonics (~145 - 400 Hz) . *In vivo* manipulation of BK_{Ca} currents using either the broad-spectrum potassium channel antagonist tetraethylammonium chloride (TEA) or the BK_{Ca} channel-specific antagonist iberiotoxin (IbTx) induced frequency-dependent changes in auditory hair cell response thresholds on the order of naturally occurring seasonal plasticity. Reproductive animals treated with either antagonist had thresholds like those recorded in non-reproductives, consistent with the role of increased BK_{Ca} channel expression facilitating higher electrical resonance in non-mammalian vertebrate hair cells. We propose that changes in BK_{Ca} channel abundance are a primary mechanism for frequency tuning plasticity in auditory hair cells among vertebrates.

Introduction

Many vertebrate sensory receptor cells are finely tuned to a portion of the total sensory spectrum from which the assembled sensory organ encodes stimuli. Variable expression of sensory transduction molecules is one way receptor cells are tuned to particular bands of the sensory spectrum. Plasticity at the receptor cell level can result from changes in expression of both opsins and photopigments determining light wavelength stimulating photoreceptors. For example, opsin gene expression changes in the rods of the European eel (*Anguilla anguilla*) as they transition from shallow water streams to deep oceanic environments, a transition induced by changes in hormones (Hope et al., 1998). Plasticity in wavelength sensitivity of photoreceptor cells in this and other fish species (Fuller et al., 2003) correlates with restricted transmission of particular wavelengths of light due to water depth and turbidity.

Water depth also differentially impacts sound transmission with cutoff frequency decreasing as depth increases (Bass and Clark, 2003). The plainfin midshipman fish (*Porichthys notatus*) displays changes in frequency encoding of both auditory hair cells in the saccule (Sisneros, 2009, Rohmann and Bass in press), the main organ of hearing in this and many other fishes (Cohen and Winn, 1967; McKibben and Bass, 1999), and the afferent neurons innervating them (Sisneros and Bass, 2003). Fish collected from shallow water environments during the reproductive season show decreased auditory hair cell response thresholds (Sisneros, 2009, Rohmann and Bass in press) as well as increased temporal precision of afferent encoding of higher frequency sounds (Sisneros and Bass, 2003) compared to animals collected from deep waters during the non-reproductive season. Such changes in

auditory sensitivity not only maximize the ability to encode sounds likely to transmit well in a given environment but also optimize detection of vocalizations produced by males nesting in the shallow intertidal zone during courtship and territory defense (Sisneros and Bass, 2003).

Electrical resonance is a primary determinant of frequency tuning in non-mammalian vertebrate auditory hair cells, with expression of large-conductance calcium-activated potassium (BK_{Ca}) channels increasing with higher resonant frequency (Fettiplace and Fuchs, 1999). It has been hypothesized that changes in BK_{Ca} channel abundance in auditory hair cells may be responsible for the auditory plasticity observed in midshipman fish (Sisneros and Bass, 2003, Rohmann and Bass in press). Thus, hair cells of animals in reproductive (shallow water/high frequency) condition would express higher levels of BK_{Ca} channels facilitating higher resonant frequencies while animals in non-reproductive (deep water/low frequency) state would have basal levels of BK_{Ca} expression and lower resonant frequencies. In support of this hypothesis, we report increased expression of genes encoding the pore-forming α-subunit of the BK_{Ca} channel in the auditory epithelium of reproductive animals as measured by quantitative real-time PCR (qPCR). Functionally reducing the number of available BK_{Ca} channels with injection of the BK_{Ca} channel antagonists TEA or IbTx in reproductive animals increased auditory thresholds in a frequency dependent manner to levels comparable to those naturally occurring in non-reproductive animals. This is the first documented mechanism of intrinsic plasticity in vertebrate auditory hair cells.

Materials and Methods

Animals

Midshipman fish have two male morphs. Type I, territorial males build and defend nests and acoustically court females with vocalizations; type II males sneak/satellite-spawn and do not perform the behaviors of type I males (Brantley and Bass, 1994). Seasonal auditory hair cell plasticity has been demonstrated in both type I males and females (Rohmann and Bass in press) and were the focus of the present study. This study included 10 females and 29 type I males. Females were collected during the non-reproductive season (November 2006; n = 5; standard length range: 12.5 - 17 cm; mean 14.6 ± 2.01 cm) by otter trawl off the coast of Washington. Reproductive females (June 2007; n = 5; standard length range: 10.5 - 12.5 cm; mean 11.6 ± 0.82 cm) and males (May – August 2010; standard length range: 12.6 - 18.6 cm; mean 15.18 ± 1.58 cm) were collected by hand from nests in the rocky intertidal zone in Tomales Bay, California. Animals were shipped to Cornell University and housed in artificial saltwater aquaria at ~16ºC and fed a diet of goldfish. The Institutional Animal Care and Use Committee at Cornell University approved all methods.

Real-Time PCR

Methods were adapted from those previously used in our laboratory (Arterbery et al., 2010a, b). Within 48 h of arrival at Cornell University, reproductive and non-reproductive adult female midshipman were first deeply anesthetized in 0.025% ethyl p-aminobenzoate (benzocaine, Sigma, St. Louis, MO, USA) dissolved in artificial seawater and tissue was then rapidly dissected, flash frozen in liquid nitrogen, and

stored at -80ºC. RNA was isolated from the combined saccular epithelia (Figure 4.1) for each individual using Trizol (Invitrogen, Carlsbad, CA, USA), DNase treated (Invitrogen), and cDNA was reverse transcribed using SuperScript III RT (Invitrogen). RNA was extracted, DNase treated, and cDNA reverse transcribed as described above for other tissues including gill, vocal muscle, and whole brain for use in primer optimization and testing of primer specificity.

Absolute (standard curve method) quantitative real-time PCR (qPCR) was conducted on cDNA using gene specific primer pairs. Because annealing temperatures and amplicon size varied between *slo1a* and *slo1b* primer pairs, each target gene was assayed separately with different housekeeper primers. Note that *slo1a* gene expression was examined prior to our laboratory developing 18s rRNA as a second housekeeper gene so only beta actin expression was measured in the *slo1a* assay. Both beta actin and 18s rRNA were measured in the *slo1b* assay. Primers were designed using the following sequences: *slo1a* (FJ269025) bp 244-264 and bp 378-398; *slo1b* (FJ269026) bp 254-277 and bp 438-461; 18s rRNA (GU586791) bp 794-819 and bp 960-983; beta actin (GQ139520) bp 62-77 and bp 201-216 (*slo1a* assay), bp 326-349 and bp 506-531 (*slo1b* assay). PCR products were sequenced by the Cornell University Life Sciences Core Laboratory Center to ensure proper sequence amplification.

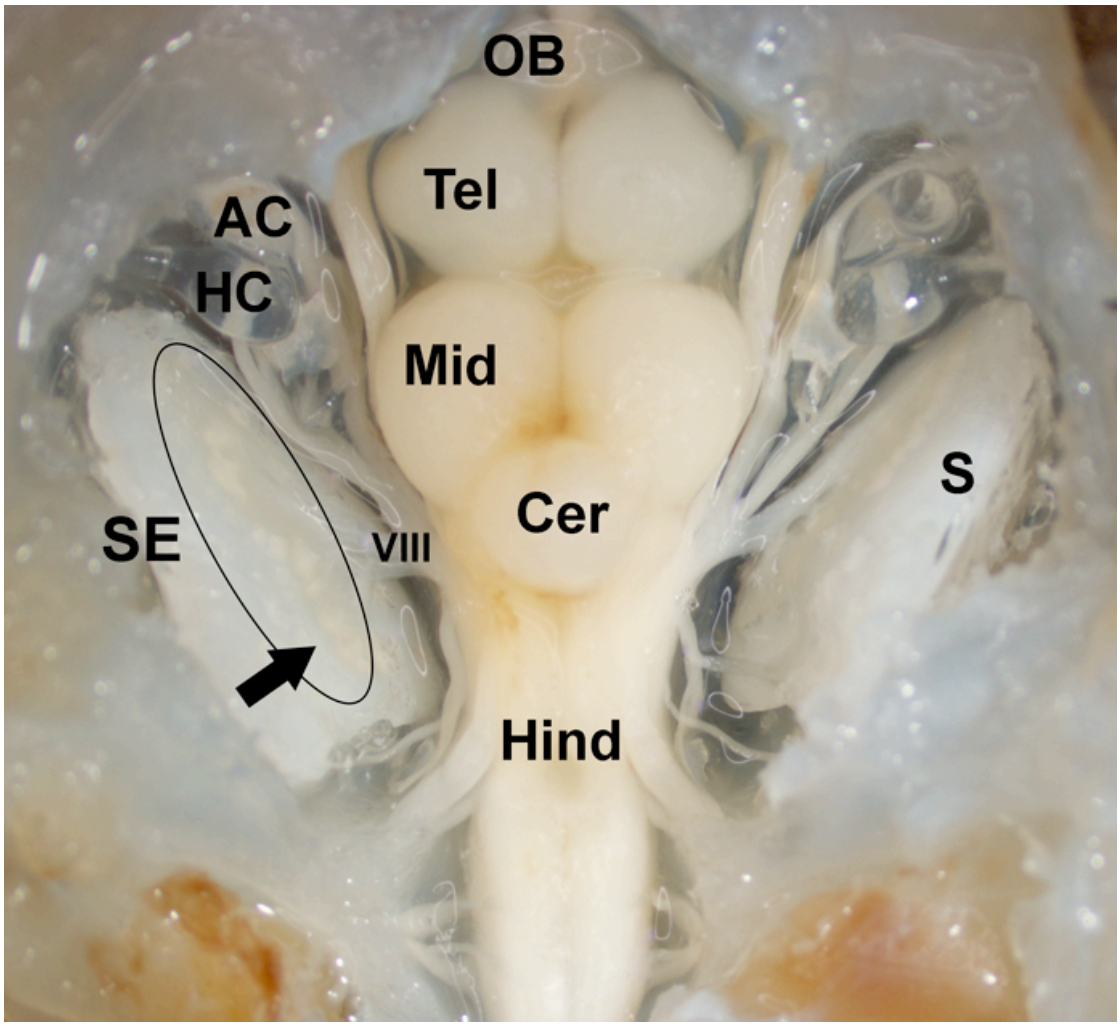


Figure 4.1
Dorsal view of brain and inner ear of plainfin midshipman fish, *Porichthys notatus*. AC, anterior canal ampulla; Cer, cerebellum; HC, horizontal canal ampulla; Hind, hindbrain; Mid, midbrain; S, saccule; SE, saccular epithelium (circled); Tel, telencephalon; VIII, eight cranial nerve. Portions of the semicircular canals were removed to more clearly visualize the eighth nerve and saccule. Arrow indicates site of injections and recordings from one saccule for *in vivo* recordings of auditory hair cell evoked potentials. While only one side is labeled, both left and right saccules were used in physiology experiments.

All real-time reactions were run in triplicate for cDNA and no template controls and standards were run in duplicate. Each well contained the following: 10µl 2xPower SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA), 2µl forward and reverse primer, 4µl H_2O, and 2µl of template. *slo1a* assay was run on an Applied Biosystems 7900HT Sequence Detection System using a 45ºC annealing temperature while *slo1b* assay was run on an Applied Biosystems Viia7 Real-Time PCR System using a 60ºC annealing temperature. Both machines were located at the Cornell University Life Sciences Core Laboratory Center. Gene copy number was determined using the standard curve method as previously described (Arterbery et al., 2010a, b) with normalized target gene copy number for *slo1a* and *slo1b* expressed using beta actin and/or 18s rRNA as reference for each cDNA sample.

Physiology

Methods for surgery, *in vivo* saccular hair cell potential recordings, and auditory stimulation follow those used previously (Rohmann and Bass, in press). The general experimental design was adopted from comparable studies of mammalian cochlea (Skinner et al., 2003). Briefly, adult type I male midshipman were anesthetized in 0.025 % ethyl p-aminobenzoate (benzocaine, Sigma) in artificial saltwater followed by intramuscular injection of pancuronium bromide (~ 0.5 mg / kg, Tocris, Ellisville, MO, USA) for immobilization and subcutaneous injection of 0.25% bupivicaine (~ 1 mg / kg, Hopsira, Inc., Lake Forest, IL, USA) with epinephrine (0.1 mg / ml, International Medication Systems, El Monte, CA, USA) for analgesia and both saccules were exposed via dorsal craniotomy (Figure 4.1). Evoked saccular potentials

were recorded as previously described (Rohmann and Bass, in press) from either non-perturbed saccules (no injection) or following injection of tetraethylammonium chloride (TEA, Sigma), iberiotoxin (IbTx, Tocris), or corresponding vehicles. Each compound was dissolved in an artificial endolymph solution made by modifying Teleost ringers solution so that Ca, Cl, K, and Na concentrations were within the range of those measured in the closely related (same family Batrachoididae) oyster toadfish, *Opsanus tau* (Ghanem et al., 2008). Artificial endolymph contained 2.88mM $CaCl_2$•$2H_2O$, 44.7mM KCl, 152mM NaCl, 15mM $NaHCO_3$, and 500μM NaH_2PO4. For IbTx, vehicle pH was adjusted to 9.0 to increase charge of IbTx molecule and 0.1% bovine serum albumin was added to reduce adhesion of IbTx to glass electrode. Compounds were injected into the saccule through a glass microelectrode inserted into the saccule that had a tip broken to a bevel (30-40 μm in diameter). A 0.5Hz square wave current (5nA TEA, 10nA IbTx) was applied to deliver compounds into the extracellular space of the saccule. Injection time ranged from 30 min (TEA, vehicle) to 1 h (IbTx, vehicle). Note that concentrations of channel antagonists (see results) reflect the concentration of antagonist in solution inside the injection electrode. The effective concentration of these compounds as they diffuse into the saccular endolymph, while unknown, is certainly lower. Evoked saccular potential recordings were used to construct threshold tuning curves as described previously (Rohmann and Bass, in press).

Statistics

All statistical analyses were performed using JMP 8 (S.A.S. Institute Inc., Cary, NC, USA). qPCR data were analyzed as described previously (Arterbery et al., 2010a, b) and physiology data were analyzed as described previously (Rohmann and Bass, in press) with injection treatment substituting as the treatment variable instead of reproductive state. Once the effect of ion channel antagonist injection into the saccule was determined, we compared the effect of injection described in the present study to the naturally occurring seasonal plasticity in saccular physiology using data from a prior study (Rohmann and Bass, in press).

Results

slo1a *mRNA is Upregulated in Saccular Epithelium During Reproductive Season*

We first used quantitative real-time PCR (qPCR) to test the hypothesis that *slo1* genes encoding the pore-forming α-subunit of BK_{Ca} channels are upregulated during the reproductive season when auditory hair cells have improved sensitivity to higher frequency sound. Primers for *slo1a* and *slo1b* were designed that produce gene-specific products (Figure 4.2). Primers for *slo1a* produced a single 154 bp product from cDNA reverse transcribed from tissues shown previously to contain only *slo1a* transcripts while failing to produce a product from tissue shown previously to contain only *slo1b* transcripts (Figure 4.2a, Rohmann et al., 2009). Primers for *slo1b* produced a single 208 bp product from cDNA reverse transcribed from whole brain which has been shown to express both *slo1* genes (Figure 4.2b, Rohmann et al., 2009). qPCR with *slo1b* primers detected minimal expression of *slo1b* in type I male vocal muscle,

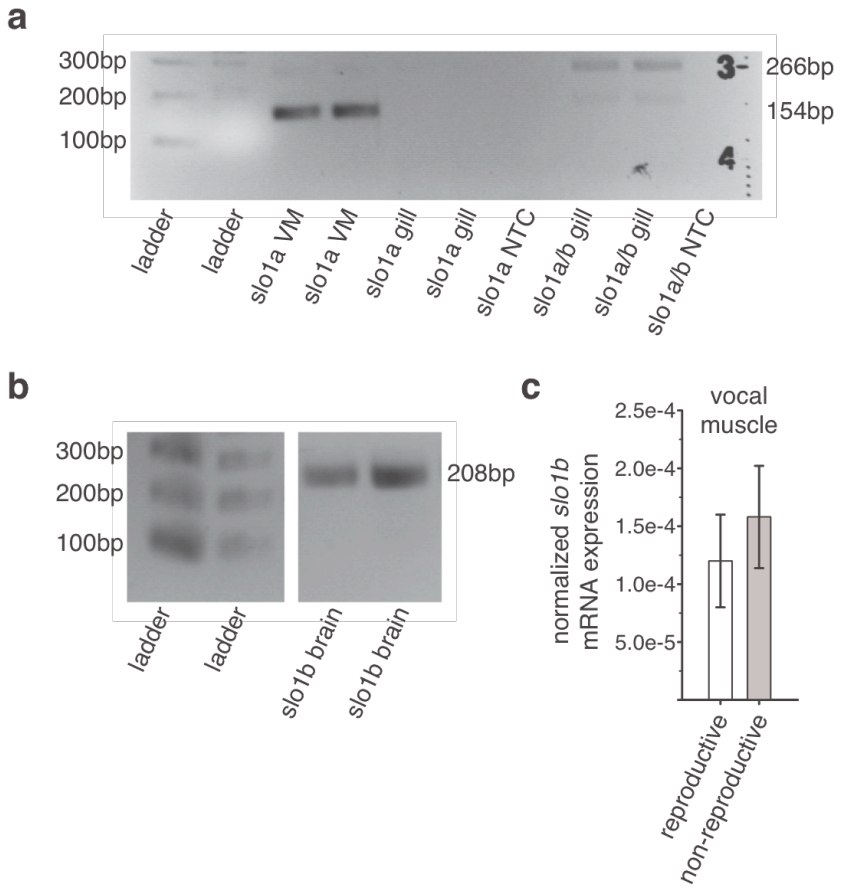


Figure 4.2

Confirmation of specificity of *slo1a* and *slo1b* qPCR primers. (a) *slo1a* qPCR primers amplify a single (154 bp) band from male vocal muscle (VM) but not from gill, tissues we have previously shown express only *slo1a* and *slo1b*, respectively. Primers that amplify both *slo1a* and *slo1b* produce the predicted 266 bp product from gill, confirming the presence of *slo1b* in this tissue. Appropriate no template controls (NTC) were run for each primer pair. (b) *slo1b* qPCR primers amplify a single (208 bp) band from brain (**c**) while detecting relatively low levels (compared to levels detected in the auditory epithelium, see Figure 4.3) of *slo1b* mRNA from male VM as predicted given the previous failure to detect *slo1b* in this tissue. Raw *slo1b* copy numbers were in the 10's-100's of copies, close to the minimum value on our standard curve (120 copies / sample). There was no significant difference in VM *slo1b* expression between seasons ($p = 0.55$, ANOVA).

a tissue shown previously to lack *slo1b* expression (Rohmann et al., 2009). PCR products from both *slo1a* and *slo1b* primers as well as for beta actin and 18s rRNA housekeeper genes were sequenced to confirm amplification of the targeted transcript.

slo1a ($p < 0.03$, ANOVA) but not *slo1b* ($p = 0.27$, ANOVA) mRNA expression normalized against beta actin mRNA expression was significantly upregulated in the saccular epithelium of reproductive females compared to non-reproductive animals (Figure 4.3). Neither beta actin mRNA (Figure 4.4a, $p = 0.67$, ANOVA) nor 18s rRNA (Figure 4.4b, $p = 0.44$, ANOVA) expression levels in the saccular epithelium differed between seasons. When normalized against 18s rRNA levels, *slo1b* mRNA expression levels were significantly elevated in saccular epithelium of reproductive compared to non-reproductive animals (Figure 4.4c, $p < 0.03$, ANOVA).

BK_{Ca} Channel Antagonists Increase Saccular Thresholds in a Frequency-Dependent Manner

If, as hypothesized, increased BK_{Ca} channel expression in reproductive animals facilitates improved encoding of higher frequency sound at the hair cell level, reducing the number of available BK_{Ca} channels should induce a non-reproductive-like auditory phenotype in reproductive animals. We first used injections of TEA, a broad spectrum potassium channel antagonist, into the saccule to test this hypothesis. There was a significant main effect of injection on saccular thresholds (Figure 4.5a, $p < 0.0001$, multi-level repeated-measures model: between-subject factor injection type) with a

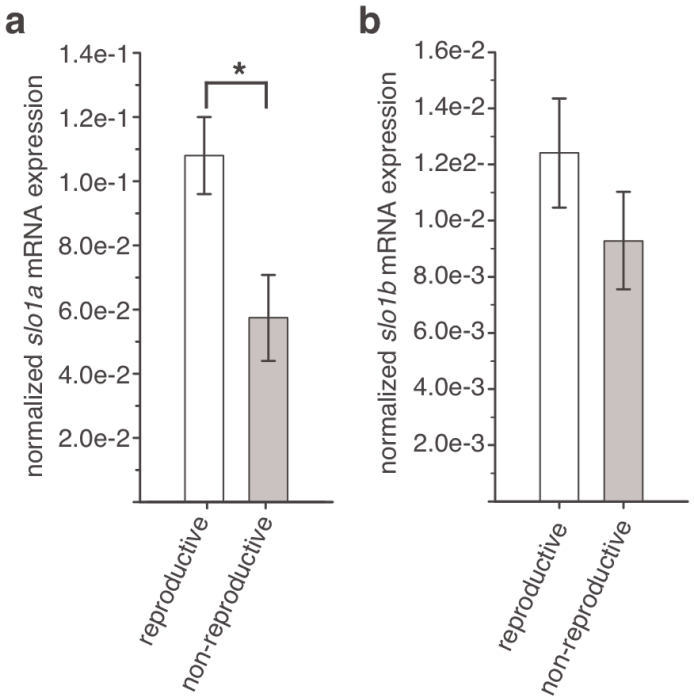


Figure 4.3
***slo1a* transcripts are upregulated in auditory epithelium during reproductive season.** (a) *slo1a* (normalized against beta actin) mRNA levels are significantly upregulated (*, $p < 0.05$, ANOVA) in reproductive animals (n = 5) compared to non-reproductives (n = 4) as revealed by qPCR. (b**)** *slo1b* (normalized against beta actin, but see Figure 4.4c) mRNA levels are stable between seasons ($p = 0.27$, ANOVA) in reproductive animals (n = 4) compared to non-reproductives (n = 5) as revealed by qPCR.

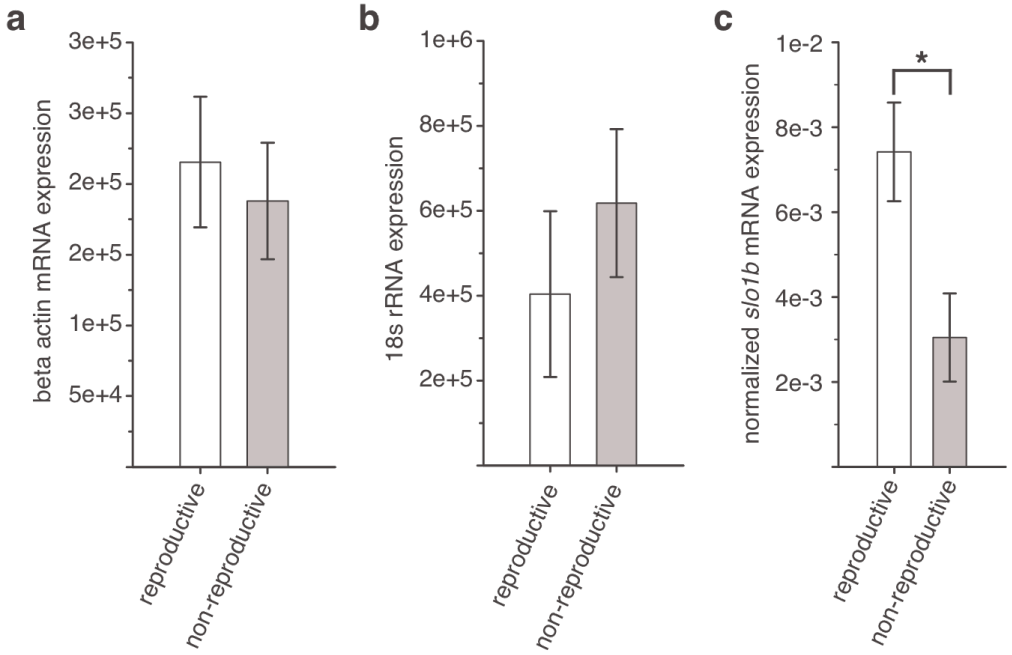
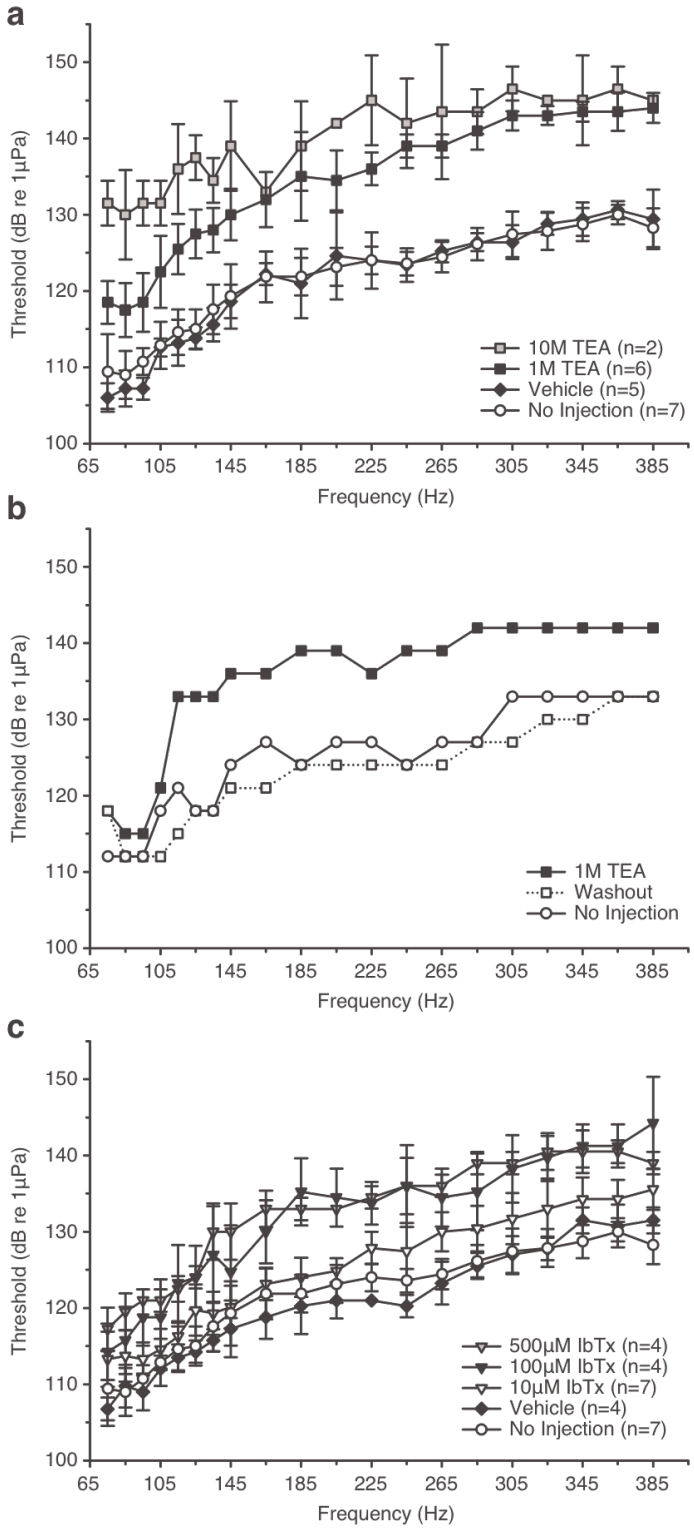


Figure 4.4
Seasonal expression patterns of 18s rRNA and beta actin mRNA qPCR housekeeper genes in the auditory epithelium. (a**)** Beta actin mRNA expression levels remain stable between reproductive (n = 4 for a-c) and non-reproductive (n = 5 for a-c) seasons as measured by qPCR ($p = 0.67$, ANOVA). (b) Similarly, 18s rRNA and expression levels remain stable between seasons ($p = 0.44$, ANOVA). (c) When normalized against 18s rRNA levels, *slo1b* mRNA levels are significantly different between seasons (*, $p < 0.05$, ANOVA, see Figure 4.3b) with levels higher during the reproductive season as is seen with *slo1a* expression (Figure 4.3a).

Figure 4.5
Injections of either TEA or IbTx into the auditory saccule increase auditory thresholds across frequencies. (a) Both 1M and 10M TEA significantly increase thresholds compared to vehicle and no-injection controls. (b) The effect of TEA is reversible with thresholds returning to a level comparable to the non-injected, contralateral saccule. (c) Both 100 and 500μM IbTx significantly increase thresholds compared to vehicle and no-injection controls. 10μM IbTx is not significantly different from either vehicle or no-injection controls.

Figure 4.5 (continued)



significant interaction between frequency and injection ($p < 0.003$). Injection of either 1M (Figure 4.5a, $p < 0.0001$, Tukey-Kramer HSD post-hoc) or 10M TEA ($p < 0.0001$, Tukey-Kramer HSD post-hoc) into the saccule produced a significant increase in thresholds compared to both vehicle and no injection controls, which did not differ significantly ($p = 0.95$, Tukey-Kramer HSD post-hoc). The effect of the two concentrations of TEA differed significantly ($p = 0.0072$, Tukey-Kramer HSD post-hoc). The effect of TEA was reversible over time, with thresholds returning to levels comparable to non-injected saccules in approximately 1 hr (Figure 4.5b).

In order to more specifically target BK_{Ca} channels, we next used injections of the specific BK_{Ca} antagonist IbTx. As was the case with the previous series of injections, there was a significant main effect of injection on saccular thresholds (Figure 4.5c, $p < 0.0001$, multi-level repeated-measures model: between-subject factor injection type) with a significant interaction between frequency and injection ($p < 0.0001$). IbTx produced significant increases in thresholds at both 100 μM (Figure 4.5c, $p = 0.0005$, Tukey-Kramer HSD post-hoc) and 500 μM ($p = 0.0003$, Tukey-Kramer HSD post-hoc) compared to vehicle controls, which did not differ from ears that were not injected ($p = 0.98$, Tukey-Kramer HSD post-hoc). 100 and 500 μM IbTx did not differ in their effects on thresholds ($p = 0.99$, Tukey-Kramer HSD post-hoc). Thresholds recorded following injections of 10 μM IbTx did not significantly differ from either vehicle ($p = 0.13$, Tukey-Kramer HSD post-hoc) or no injection ($p = 0.18$, Tukey-Kramer HSD post-hoc) controls.

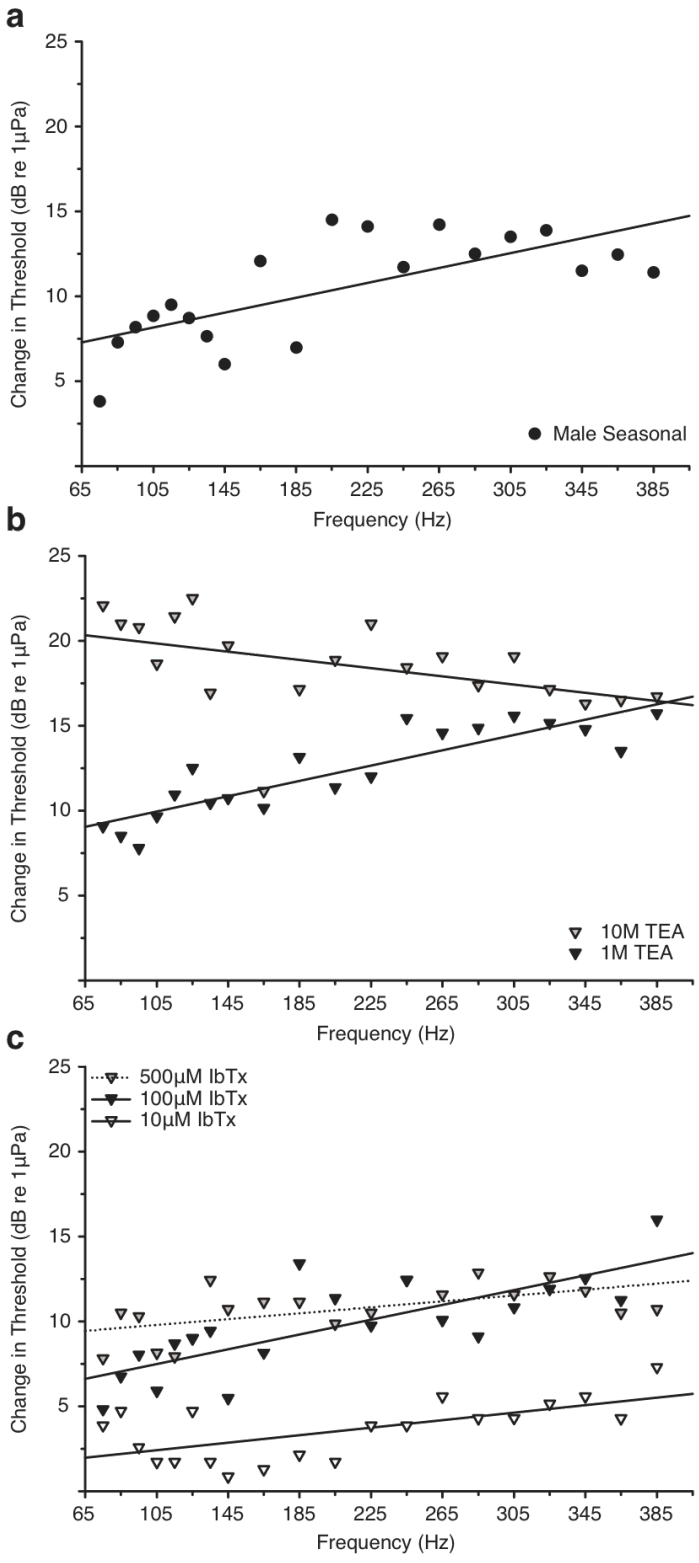
One defining feature of natural saccular hair cell plasticity is its frequency dependence (Figure 4.6a, data from Rohmann and Bass in press) with differences in thresholds greatest at higher frequencies. Several doses of BK_{Ca} channel antagonists that increased thresholds in reproductive animals did so in a frequency dependent manner with the greatest change occurring at higher frequencies including 1M TEA (Figure 4.6b, $p<0.0001$, $R^2=0.58$), and all three doses of IbTx (Figure 4.6c, 10μM – $p=0.0025$, $R^2=0.41$, 100μM – $p<0.0001$, $R^2=0.60$, 500μM – $p=0.0079$, $R^2=0.33$). The higher dose of TEA (10M) used resulted in a reversed relationship between change in threshold and frequency (Figure 4.6b, $p=0.04$, $R^2=0.22$) consistent with the increase in thresholds at frequencies ≤ 145 Hz beyond those observed to occur naturally (Figure 4.5a). As was the case with natural seasonal plasticity (data from Rohmann and Bass in press) 1M TEA and both 100 and 500 μM IbTx produced threshold shifts that are greater at frequencies above 145Hz than below (Figure 4.7, 1M TEA – $p < 0.0001$, 100 μM IbTx – $p = 0.0002$, 500 μM IbTx – $p = 0.006$, ANOVA).

BK channel Antagonists Induce Changes Comparable to Natural Seasonal Plasticity

Both 1M TEA and 100-500μM IbTx injections into the saccule of reproductive animals increase saccular thresholds in a frequency-dependent manner similar to naturally occurring seasonal plasticity in adult midshipman of both sexes. When either 1M TEA (Figure 4.8a) or 500μM IbTx (Figure 4.8b) threshold tuning curves are compared to reproductive and non-reproductive animals, both antagonists produce thresholds that are significantly different from reproductive animals (TEA vs.

Figure 4.6
Seasonal and pharmacologically-induced changes in auditory thresholds are frequency-dependent. (a) Amplitude of seasonal changes in hair cell thresholds is positively correlated with frequency ($p=0.0005$, $R^2=0.50$, data from Rohmann and Bass in press). (b) 1M TEA ($p<0.0001$, $R^2=0.58$) and (c) three doses of IbTx (10μM – $p=0.0025$, $R^2=0.41$, 100μM – $p<0.0001$, $R^2=0.60$, 500μM – $p=0.0079$, $R^2=0.33$) all show a similar correlation between change in threshold and frequency. At a higher dose (10M) of TEA, the relationship between change in threshold and frequency is reversed ($p=0.04$, $R^2=0.22$).

Figure 4.6 (continued)



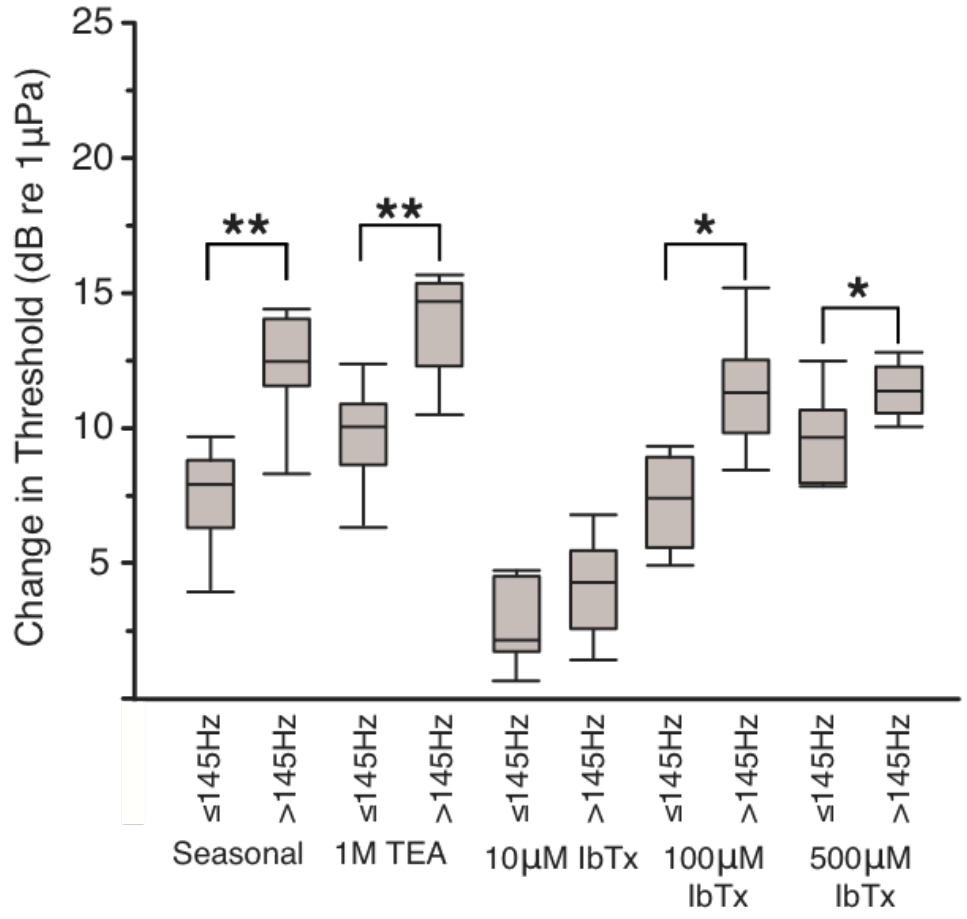


Figure 4.7
Seasonal and pharmacologically-induced changes in auditory thresholds are greatest at frequencies above 145Hz. Seasonal plasticity in male midshipman is greatest at frequencies above 145Hz (data from Rohmann and Bass in press). 1M TEA and 100 and 500µM IbTx induce changes from baseline that are similarly greater at frequencies above 145Hz. A lower dose of IbTx (10µM) has no significant difference in its effect at frequencies above or below 145Hz. * denotes a significant difference between groups ($p<.01$). ** denote a significant difference between groups ($p<0.0001$).

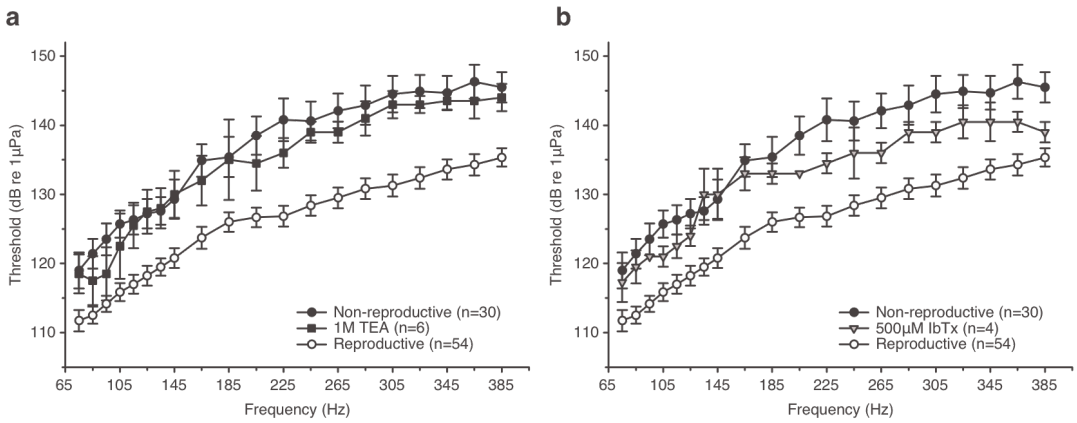


Figure 4.8
Both TEA and IbTx shift auditory thresholds from a reproductive to non-reproductive like state. Injections of either (a) 1M TEA or (b) 500µM IbTx into the auditory saccule of reproductive animals shifts thresholds such that they are significantly different from non-manipulated reproductive animals but statistically indistinguishable from non-reproductive animals. Data for reproductive and non-reproductive animals are combined recordings from both males and females from Rohmann and Bass (in press). The same data is plotted in both (a) and (b) to more clearly illustrate comparisons with either (a**)** TEA or (b) IbTx.

reproductive $p = 0.0002$, IbTx vs. reproductive $p < 0.05$, Tukey-Kramer HSD post-hoc test) but not different from non-reproductive animals (TEA vs. non-reproductive – $p = 0.56$, IbTx vs. reproductive – $p = 0.27$, Tukey-Kramer HSD post-hoc test).

Discussion

Our data support the hypothesis that naturally occurring plasticity in auditory hair cell physiology coincides with changes in expression of the *slo1a* gene encoding the α-subunit of BK_{Ca} channels. Pharmacological manipulations in which antagonists reduce the number of functioning BK_{Ca} channels produce threshold tuning curves on the opposite range of natural hair cell plasticity from those treated with vehicle or no injection controls. Physiology data supports the hypothesis that increased *slo1a* in the auditory epithelium of reproductive animals results in increased BK_{Ca} channel expression facilitating improved encoding of high frequency sounds. To our knowledge this is the first study to demonstrate a molecular mechanism for long-term (weeks to months) plasticity in auditory hair cells.

Seasonal plasticity of auditory (saccular) hair cell physiology has been demonstrated in both female (Sisneros, 2009, Rohmann and Bass in press) and type I male midshipman (Rohmann and Bass in press). Given the lack of sex differences in physiology, we hypothesize that the mechanism(s) of plasticity is shared across all individuals in this species. Our qPCR experiments used females because peripheral auditory plasticity had only been demonstrated in females at the time this experiment began (Sisneros and Bass, 2003; Sisneros, 2009). Upon discovery of comparable plasticity in type I males (Rohmann and Bass in press) we focused physiology

experiments on males because of greater availability. Though conducted in different sexes, both molecular and physiology results are consistent with our broader hypothesized mechanism for hair cell plasticity. While all available data argues against it, there remains the possibility that there is a sex difference in either the mechanism(s) of plasticity or the degree to which various factors contribute to plasticity in either sex.

Another as yet unanswered question is whether plasticity at the hair cell level is steroid-dependent as has been demonstrated for the afferents innervating them (Sisneros et al., 2004). Hair cell plasticity correlates with natural changes in circulating steroids as animals transition from non-reproductive to reproductive condition and may ultimately be driving both natural and steroid-induced plasticity at the afferent level (Rohmann and Bass in press). If confirmed, steroid-dependent changes in auditory hair cells would constitute yet another case of steroid-dependent changes in a hair cell based sensory systems. Interestingly, changes in ion channel expression have been proposed as the mechanism for steroid-dependent plasticity of frequency sensitivity in electroreceptor (modified hair cells) tuning in weakly electric fishes (Zakon and Meyer, 1983; Bass and Hopkins, 1984). Divergent levels of *slo1a* expression may be due to changes in expression levels in existing hair cells or turnover of hair cells as has been demonstrated in other fishes (Corwin, 1983), with new cells added with different electrical properties than preexisting cells. Increases in *slo1a* expression may be due to addition of new hair cells with higher *slo1a* expression and higher resonant frequencies.

While *slo1a* mRNA levels varied significantly between non-reproductive and reproductive animals when normalized against beta actin mRNA levels, we did not see significant changes in levels of *slo1b*. However, when we normalized *slo1b* levels against 18s rRNA expression we saw a significant difference similar to that observed for *slo1a*. Both beta actin mRNA and 18s rRNA levels were stable between the two populations of animals measured (**Fig. 3a,b**), supporting their use as housekeeper genes. We have noticed similar patterns in other qPCR studies conducted on auditory epithelium with statistical significance being achieved when normalized against one housekeeper (usually 18s) but not the other but, as was the case in this study, never resulting in opposite trends (D. J. Fergus, D. L. Deitcher, and A. H. Bass, unpublished observations). Future studies will focus on determining whether *slo1b*, like *slo1a*, differs in expression levels between seasons. If *slo1b* expression does remain stable as our present data suggest, it may indicate varying functions between these duplicate *slo1* genes. Data from midshipman suggests tissue-specific subfunctionalization of these two genes outside of the nervous system (Rohmann et al., 2009). In auditory hair cells *slo1a* may preferentially encode channels with faster kinetics or greater amplitudes such as are found across the *slo1* expression gradient in turtle and chicken hair cells (Jones et al., 1999; Ramanathan et al., 1999). Divergent regulation of *slo1a* and *slo1b* expression may provide information on the transcriptional regulation of these genes and the signaling factors that may mediate hair cell plasticity at the molecular level.

Without *in situ* hybridization studies to confirm localization of *slo1a* expression in the saccular epithelium, we cannot rule out the possibility that changes

in *slo1a* mRNA levels observed with qPCR are not due, at least in part, to expression in cells other than hair cells, including ganglion cells (Skinner et al., 2003). Changes in BK_{Ca} channel expression in ganglion cells could further contribute to afferent plasticity in addition to changes due to plasticity in hair cell input. Future immunohistochemistry studies will localize BK_{Ca} channel expression to address this issue and aim to confirm that changes in *slo1a* mRNA levels parallel changes in *slo1a* protein levels. Physiological data support this assumption and, unlike qPCR measures, the recording method used here measured evoked potentials only from hair cells, thus allowing us to measure the effects of BK_{Ca} channel antagonists on hair cells alone (see Rohmann and Bass in press for discussion of methods).

One interesting characteristic of both natural plasticity and BK_{Ca} antagonist-induced changes is that hair cell responses differ in both amplitude (threshold) and frequency of sounds due to what we propose is a single molecular mechanism. This reflects the dual role of BK_{Ca} currents in encoding both the alternating (AC) and direct (DC) current components of hair cell membrane potentials (Russell and Sellick, 1978; Dallos, 1985). As discussed earlier, increased BK_{Ca} channel expression correlates with higher electrical resonant frequency in non-mammalian vertebrate hair cells (Fettiplace and Fuchs, 1999). Cells with higher electrical resonance also have larger amplitude BK_{Ca}-like currents (Art et al., 1986). In our experiments, reducing BK_{Ca} currents in the saccule led to decreases in best resonant frequencies. As our measuring technique indicates that a large population of cells undergoes this shift, it may result in an increase in thresholds at higher frequencies where cells that express more BK_{Ca} channels are more susceptible to the effects of BK_{Ca} antagonists. Within the range of

frequencies studied, BK_{Ca} channels are a primary source of large amplitude, rapid outward current in fishes and other vertebrates, including mammals (Steinacker and Romero, 1991, 1992; Kros et al., 1998; Fettiplace and Fuchs, 1999). BK_{Ca} channel antagonists may further increase thresholds by reducing this large outward current in all hair cells regardless of resonant frequency, as the microphonic recording technique used in our experiments measured the evoked potential generated by cation influx through mechanotransduction channels as well as outward (K+) and inward (Ca2+) currents at the opposite end of the hair cell (see Nicolson et al., 1998 for methodology discussion). The effects of BK_{Ca} channel antagonists on these two processes may combine to change hair cell thresholds across all frequencies, but with a greater effect at higher frequencies.

The above explanation for auditory threshold changes is of particular interest as it has the potential to apply across vertebrates regardless of whether frequency tuning of the auditory division of the inner ear is achieved through either electrical or mechanical tuning mechanisms. While mammals rely on mechanical means of frequency tuning in the inner ear, BK_{Ca} currents are still a primary outward current in many inner hair cells (IHC, Kros et al., 1998) and outer hair cells (OHC, Wersinger et al., 2010). BK_{Ca} channel antagonists block a large-amplitude rapid outward current in both non-mammalian (Art et al., 1986) and mammalian IHCs (Kros et al., 1998; Skinner et al., 2003) hair cells. *slo1* knockout mice exhibit early onset hearing loss linked to OHC dysfunction (Ruttiger et al., 2004) as well as deficits in IHC and afferent function (Oliver et al., 2006).

Mouse models of Turner syndrome (45,X) show early onset high frequency hearing loss and hair cell pathology (Hultcrantz et al., 2000) similar to that reported for *slo1* knockout mice consistent with the regulation of mouse *slo1* expression by estrogen (Zhu et al., 2005; Kundu et al., 2007). This pattern of hair cell degeneration and hearing loss most prominently at higher frequencies mirrors changes observed in *slo1* knockout mice and is consistent with the frequency-dependent role of BK_{Ca} channels mediated efferent-OHC synapses (Wersinger et al., 2010). Much as steroids may act to modify expression of BK_{Ca} channels to facilitate high frequency hearing in animals like midshipman that utilize electrical resonance, the same molecular mechanism may promote high frequency hearing in mammals in which BK_{Ca} channels play a role that at first appears divergent because of the derived function of outer hair cells but is ultimately a deeply conserved functional role in auditory plasticity. Examination of BK_{Ca} expression in Turner syndrome mice as well as estrogen receptor knockout lines could shed light on the mechanism of steroid modulation of auditory plasticity in mammals including humans in which steroids play a role in alleviating the effects of age-related hearing loss, presbycusis (Hultcrantz et al., 2006).

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CHAPTER 5

BK_{Ca} CHANNEL FACILITION OF HIGH FREQUENCY HEARING ACROSS VERTEBRATES

As discussed in the preceding chapter, the identification of BK_{Ca} expression as a mechanism for auditory plasticity at the hair cell level in midshipman fish raises the question of whether this phenomenon might generalize across vertebrates. Of particular interest, as addressed in the introductory chapter, is how this mechanism for auditory plasticity might interact with steroid hormones to modulate ontogenetic changes in high frequency hearing in humans as it relates to age-related hearing loss, presbycusis. While the previous chapters admittedly do not directly address whether changes in steroids are either necessary or sufficient to induce the natural molecular and physiological changes in auditory hair cells characterized in midshipman, it is ultimately the interplay between steroid levels and auditory function that was the impetus for these experiments and it is in this context in which future experiments will be focused. This chapter will provide further discussion on the known role of steroids in ontogenetic changes in mammalian hearing as well as the role of BK_{Ca} channels in mammalian hair cell physiology. Future experimental avenues will be discussed that aim to merge these two fields of research together in light of discoveries from midshipman fish.

Steroids and Ontogenetic Changes in Human Audition

In humans, one of the most common communication disorders and form of hearing deficits is presbycusis, age-related hearing loss (Jonsson et al., 1998). Jonsson et al.

(1998) showed that presbycusis is worse for men than women of the same age range (70-75 years-of-age) and pronounced at frequencies above 2kHz and while men exhibit the greatest hearing loss between 2 and 4kHz, their female counterparts have the greatest hearing deficits at higher frequencies, ranging from 4-8kHz. Postmenopausal women with higher estrogen levels have less hearing loss than those with low levels (Kim et al., 2002). Similarly, women who take estrogen as postmenopausal hormone replacement therapy (HRT) show lower hearing thresholds (less hearing loss) than those without HRT (Kilicdag et al., 2004). Women with Turner syndrome (45,X), a genetic disorder characterized by low levels of estrogen, experience premature presbycusis compared with age-matched (46,XX) female counterparts (Hultcrantz et al., 1994).

Together, the above studies suggest that estrogen plays a role in preserving high frequency hearing during aging. Estrogen may act locally within the peripheral auditory system as cells in the inner ear of mammals, including humans, express estrogen receptors (ER) alpha (α) and beta (β) (Stenberg et al., 2001; Stenberg et al., 2002). Because of the potential health risks of HRT (Rossouw et al., 2002), maintaining high levels of circulating estrogen is not a viable option for presbycusis prevention. As a result, it is important to understand the mechanism(s) by which estrogen modulates high frequency hearing in order to limit the effects of this common communication disorder in a population that continues to age and in which this disorder is likely to become an increasing public health concern.

Auditory Deficits in Mouse Models with Altered Estrogen Signaling

As discussed in Chapter 4, mouse models have been developed to begin to study the roles of steroids, specifically estrogen, and BK_{Ca} physiology in audition but without overlap between these two lines of investigation. Mouse models of Turner syndrome (45,X) show early onset high frequency hearing loss and hair cell pathology (Hultcrantz et al., 2000). Specifically, there is a marked loss of outer hair cells (OHCs) in the basal (high frequency) turn of the cochlea coinciding with decreased distortion product otoacoustic emissions (DPOAE) indicative of deficits in OHC function (Hultcrantz et al., 2000). Both OHCs and inner hair cells (IHCs) are ultimately lost in Turner syndrome mice resulting in physiological deficits as measured by auditory brainstem responses. The observation that expression of ERβ decreases, while ERα levels remain comparable to control mice, indicates that deficits in estrogen signaling in Turner syndrome mice may preferentially affect ERβ signaling (Stenberg et al., 2002).

As the name implies, Turner syndrome is a broader disease that affects a variety of estrogen-dependent pathways. More focused experiments on the role of estrogen signaling in mammalian peripheral auditory physiology come from ER knockout mice. ERβ knockout mice are deaf by 12 months of age and show death of both IHCs and OHCs in the basal (high frequency) turn of the cochlea (Simonoska et al., 2009) where hair cell loss is most pronounced in Turner mice (Hultcrantz et al., 2000). The loss of hair cells in these animals was attributed to downregulation of many critical genes expressed in hair cells that were previously shown to be downregulated in other tissues in ERβ knockout mice as measured by microarray (Lindberg et al., 2003). The observation that both Turner mice and ERβ knockout

mice show similar anatomical and physiological deficits in the peripheral auditory system suggests that ERβ signaling pathways may be key in both animal lines and ultimately necessary for maintenance of normal high frequency hearing.

Both the estrogen dependence of ERβ expression in mice (Stenberg et al., 2002) and its potential role in high frequency hearing (see above) are consistent with ongoing studies in midshipman that have demonstrated upregulation of both ERβ1 and ERβ2 transcripts in the auditory epithelium of reproductive compared to non-reproductive females (Fergus DJ, Deitcher DL, and Bass AH, unpublished observations). Pre-nesting increases in circulating estrogen levels (Sisneros et al., 2004) may be responsible for this increase in ERβ expression in midshipman. Both ERβ1 and ERβ2 immunoreactivity has been localized to auditory saccular hair cells in midshipman (Fergus DJ, Deitcher DL, and Bass AH, unpublished observations) indicating that ERβ signaling is upregulated at the same time that *slo1a* expression is increasing within the saccule. Estrogen action through ERs may directly regulate *slo1* expression in midshipman as has been shown for mammalian *slo1* both *in vitro* (Kundu et al., 2007) and *in vivo* (uterus: Holdiman et al., 2002; myometrium: Zhu et al., 2005). Experiments to test whether estrogen is sufficient to induce upregulation of both *slo1* and ERβ transcripts in the auditory epithelium of non-reproductive midshipman are currently under way. If estrogen regulation of BK_{Ca} expression is critical for auditory hair cell function, animal models in which BK_{Ca} expression has been perturbed should produce similar phenotypes as Turner and ERβ knockout mice.

Auditory Deficits in BK_{Ca}α -/- Mice

As previously mentioned, BK_{Ca} currents are a primary outward current in both mammalian IHCs (Kros et al., 1998; Skinner et al., 2003) and OHCs (Wersinger et al., 2010) and, as such, retain a functionally important role in mammalian hair cell physiology despite the lack of electrical tuning in mammalian hair cells (Fettiplace and Fuchs, 1999). From post-natal day 12, when the auditory system of mice becomes functional, BK_{Ca} channels are expressed in the membranes of both IHCs and OHCs (Hafidi et al., 2005) with the adult phenotype of K^+ channel currents established by post-natal day 19 (Kros et al., 1998). Interestingly, BK_{Ca}-immunoreativity in OHCs falls along a tonotopic gradient reminiscent of studies of the basilar membrane (cochlea homologue) in chickens and turtles (Fettiplace and Fuchs, 1999) with BK_{Ca}-immunoreactivity greatest in OHCs in the basal (high frequency) end of the cochlea (Hafidi et al., 2005; Engel et al., 2006; Wersinger et al., 2010). While the presence of clearly defined populations of outer and inner hair cells is a derived trait in mammals, OHCs appear to have retained the ancestral condition of tonotopic expression of BK_{Ca} channels. This raises the exciting possibility that a BK_{Ca} mediated mechanism for high frequency auditory plasticity may yet be retained in mammals despite the anatomical and physiological differences in means of frequency encoding between mammals and non-mammalian vertebrates such as midshipman.

Hafidi et al. (2005) hypothesized that, since BK_{Ca}-immunoreactivity was concentrated in OHCs at the basal end of the cochlea, both BK_{Ca} and SK_{Ca} currents may mediate the efferent cholinergic synapse. This hypothesis was later supported in that BK_{Ca} channels, expressed at higher levels in high frequency encoding OHCs, mediate efferent cholinergic synapses and are activated by Ca^{2+} entry through Ca^{2+}

permeable α9α10-containing nicotinic cholinergic receptors (nAChRs), while $SK_{Ca}2$ channels serve a similar function in lower frequency encoding OHCs (Wersinger et al., 2010). The role of BK_{Ca} channels in mediating efferent input specifically in high frequency OHCs raises the possibility that BK_{Ca} in the mammalian ear may serve as a mechanism for preferentially modulating high frequency hearing.

Investigations using $BK_{Ca}\alpha$ (*slo1*) -/- knockout mice have done a great deal to confirm this possibility. Several studies of $BK_{Ca}\alpha$ mice have shown deficits in hearing, especially at frequencies greater than 8 kHz (Ruttiger et al., 2004; Engel et al., 2006; Oliver et al., 2006; but see Pyott et al., 2007). $BK_{Ca}\alpha$ mice display decreased DPOAEs, indicative of declining OHC function, by 8 weeks of age (Ruttiger et al., 2004) followed closely by OHC death in the basal (high frequency) end of the cochlea (Engel et al., 2006). While OHCs are undergoing significant structural and physiological signs of decay, IHCs do not appear to undergo any marked changes in either structure or graded potentials (Ruttiger et al., 2004), suggesting that hearing loss in these animals is at least initially through deficits in OHC function.

Prior to the loss of KCNQ4 expression in degenerating OHCs, which appears to be one of the hallmarks of OHC death, there is a breakdown of the efferent-OHC synapse and decreased expression of the motor protein prestin in basal (high frequency) OHCs (Ruttiger et al., 2004). The degradation of the efferent synapse is consistent with the critical role of BK_{Ca} currents in modulating efferent inhibition of OHCs (Wersinger et al., 2010). The model put forth by Ruttiger et al. (2004) suggests that the lack of BK_{Ca} currents prevents the OHC from repolarizing and allows voltage-gated Ca^{2+} channels to remain open for prolonged periods leading to Ca^{2+}

excitotoxicity. Decreased prestin expression may further underlie deficits in OHC function as prestin is the motor protein that converts electrical activity in OHCs into changes in cell length allowing OHCs to actively control amplification of vibrations along the cochlea (Dallos and Fakler, 2002).

Examining BK_{Ca} Expression in ERβ Mice

While mice deficient in either estrogen signaling or BK_{Ca} channel expression have auditory deficits that ultimately result in cell death within the same population of hair cells, it remains to be seen whether this is pure coincidence or whether, as we hypothesize for midshipman, estrogen signaling indeed regulates BK_{Ca} expression in hair cells as it does in other tissues (e.g., Zhu et al., 2005). Future experiments in which BK_{Ca} currents and protein expression are measured in ERβ knockout mice leading up to the onset of measurable auditory deficits and cellular degeneration will test this hypothesis. If BK_{Ca} channel expression is maintained by estrogen signaling and its loss is the driving force behind accelerated high frequency hearing loss in ERβ knockout mice, we predict that the tonotopic gradient of BK_{Ca} channel expression should degrade prior to morphological and physiological changes in OHCs with changes greatest in OHCs within the basal (high frequency) end of the cochlea. Such a discovery would hold the potential to allow for medical intervention to prevent degeneration of high frequency OHCs through targeted gene therapy that would avoid the complications of systemic estrogen therapy.

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